POLLINATOR-DRIVEN DIVERGENCE AMONG POPULATIONS OF A SELF-FERTILIZING LILY, *HESPERANTHA COCCINEA* (IRIDACEAE)

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Submitted in fulfilment of the academic requirements for the degree

Doctor of Philosophy

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Pietermaritzburg

January 2021

ABSTRACT

Two major trends in floral evolution – pollinator shifts and the evolution of autonomous self-fertilisation – are generally considered alternative evolutionary responses to pollen-limitation of plant reproductive success. However, pollinator-specialised species often are also autofertile. The apparent contradiction of "opposing contrivances" for attracting pollinators and reproducing independently of them, may represent an optimal Best-of-Both-Worlds strategy whereby delayed self-fertilisation provides reproductive assurance in unpredictable pollination environments.

In this thesis, I demonstrate pollinator-driven divergence among autofertile populations of *Hesperantha coccinea* (Iridaceae) based on evidence of local adaptation to different pollinators and experimental quantification of the contributions of pollinators and autonomous self-fertilisation to reproductive success. Floral colour, morphology, orientation and nectar characteristics differ between populations pollinated by a butterfly or a long-proboscid fly. Reciprocal translocation of plants, assessment of pollinator effectiveness and preference experiments demonstrate that this intraspecific divergence involved adaptation to the morphology and preferences of the locally-dominant pollinators at low and high elevations, creating a geographic mosaic of floral variation.

Despite this divergence, reproduction by both ecotypes involves a combination of pollinator-mediated outcrossing and autonomous self-fertilisation. Hand-pollinations showed self-compatibility and high autofertility in both ecotypes. Nevertheless, analysis of SSR markers revealed mixed selfing and outcrossing in populations of both colour forms. Most autonomous self-pollination occurred late during a flower's lifespan, as expected for Best-of-Both-Worlds reproduction. Furthermore, similar performance of selfed and outcrossed progeny from three populations in a greenhouse indicated little genetic cost of selfing. Emasculation experiments showed extensive variation in the relative contributions of autonomous self-pollination and pollinators to fecundity among populations and flowering seasons. Overall, pollinator activity and autonomous self-fertilisation accounted for 75% and 25% of fecundity, respectively. The contribution of autonomous self-fertilisation varied among populations from zero to more than 90% of seed set and differed within populations between years by an average of 30%. The relative importance of pollinators and autonomous self-fertilisation did not vary geographically in relation to proximity to range edge, flower number, size, or herkogamy. This independence identifies autonomous self-fertilisation as part of a stable Best-of-Both-Worlds strategy employed by *H. coccinea* to contend with

unpredictable pollination. Weak inbreeding depression in combination with conditions otherwise consistent with Best-of-Both-Worlds reproduction suggests that the importance of siring advantages of pollinator-mediated pollen transfer have been underestimated in these systems.

PREFACE

The experimental work described in this dissertation was carried out in the Republic of South Africa under the auspices of the School of Life Sciences, University of Natal, Pietermaritzburg, from March 2010 to May 2020 under the supervision of Professor Steven D. Johnson and co-supervision of Professor Lawrence D. Harder.

This thesis represents original work by the author and has not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

Ruth Cozien

2 January 2021

I certify that the above statement is correct

Professor Steven D. Johnson (supervisor)

As the candidate's supervisor I have approved this thesis for submission.

Professor Steven D. Johnson (supervisor)

Professor Lawrence D. Harder (co-supervisor)

DECLARATION 1 - PLAGIARISM

I, Ruth Jenny Cozien declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.
2. This thesis has not been submitted for any degree or examination at any other university.
3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.
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DECLARATION 2 - PUBLICATIONS

Chapters 2 to 5 of this thesis are manuscripts which have either been submitted or are intended for submission to appropriate journals. In all cases, author contributions are as follows:

RJC, LDH and SDJ conceived the study. RJC collected and analysed the data with assistance from LDH and SDJ. RJC wrote the manuscripts with editorial inputs from SDJ and LDH.

I certify the above statement is correct

Ruth Cozien

Professor Steven D. Johnson (supervisor)

Professor Lawrence D. Harder (co-supervisor)

ACKNOWLEDGEMENTS

Numerous people have supported me during this project.

Most importantly, I have had two exceptional supervisors. Steve's enthusiasm is contagious and indomitable. Few students are privileged to experience the stimulation, freedom and resources to follow our curiosity that working in his research group provides. Lawrence's expertise and meticulousness are fortunately accompanied by exceptional patience. I am deeply grateful to both my supervisors for everything I have learned through working with them.

This work was funded by the National Research Foundation of South Africa. South African National Parks, Ezemvelo KwaZulu-Natal Wildlife, the Eastern Cape Department of Economic Development and Environment Affairs and the Mpumalanga Tourism and Parks Agency granted research permits and use of their research facilities. Private landowners including Peter Carr, Robyn Conroy, Juergen Goosen, Russel and Jan Hobday and Danie Zietsman provided access to field sites.

Many colleagues have provided technical assistance and friendship in Best-of-Both-Worlds combinations. Genevieve Theron produced artwork for figures in Chapters 4 and 5 and enforced tea breaks. Isabel Johnson mapped ever increasing numbers of study populations with ArcGIS and shared her always top quality chocolate. Micheal Whitehead taught me how to use GenAlEx and drive sideways though mud. Jana Jersakova and Craig Peter provided templates for insect vision models, Adam Shuttleworth helped me understand them. Samson Tesfay and Sandy-Lynn Steenhuisen showed me how to run HPLC nectar analyses. Rene Veikondis and the team at the Central Analytical Facility provided training, ran samples and were unfailingly helpful answering questions. Tanya Karalic, Renny Noble, Pat Joubert, Ziyanda Ndlovu and Charmaine Ahrens provided efficient administrative assistance. Peter Mutabazi and Alison Young and the team at the UKZN Botanical Gardens helped set up and maintain the common garden project. Dara Stanley, Carolina Diller, Terence Suinyuy and James Rodger shared ideas and coffee. Many people assisted with data collection, often voluntarily spending hours dabbling their toes in streams and watching butterflies and flowers: thanks in particular to Su Cozien, Stuart Hall and Nina Hobbhahn for tolerating these punishing schedules. Nina Hobbhahn got me into this and her humour, encouragement and perspective played a major role in getting me through it. Timo van der Niet has provided diverse, endless and invaluable support during this project. Thank you, I could not have done this without you.

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CHAPTER 1: THESIS INTRODUCTION

Background

The spectacular variety of floral traits among the c. 300 000 species of angiosperms reflects variation in reproductive systems (Barrett et al. 1996). Major drivers of floral variation include shifts in pollination system, such as transitions to a novel pollinator and from predominantly pollinator-mediated outcrossing to autonomous self-fertilisation (Stebbins 1970). Shifts in pollination system are associated with approximately one quarter of divergence events in angiosperm groups for which data are available (van der Niet and Johnson 2012). These transitions are associated with changes in floral traits involved in pollinator attraction and morphological fit to different pollinators (Rosas-Guerrero et al. 2014). Shifts to selfing, among the most frequently travelled evolutionary pathways in flowering plants (Stebbins 1974, Igic et al. 2008), are associated with the evolution of "selfing syndrome" traits, including small flowers with reduced stigma-anther separation and low pollen:ovule ratios (Sicard and Lenhard 2011). Both pollinator changes and shifts to selfing are driven by selection to alleviate limitations to reproductive success that result from changes in local pollinator abundance or effectiveness, and the associated changes in pollination systems and floral traits may occur within a few generations (Bodbyl Roels and Kelly 2011, Gervasi and Schiestl 2017).

Despite the commonalities in their causes and consequences, transitions to selfing and different pollinator are generally considered alternative evolutionary trajectories (Harder 1996). However, the prevalence of showy selfers, species that reproduce by a combination of pollinator-mediated reproduction and autonomous self-fertilisation, implies that pollinator adaptation and selfing are not mutually exclusive (Fenster and Martén-Rodríguez 2007). Clearly, selfing and pollinator adaption can be maintained together and their consideration as alternative evolutionary pathways may limit understanding of floral evolution. In particular, if the selection that maintains pollinator adaptation in showy selfers can also facilitate pollinator shifts in autofertile species, the prevalence of showy selfers implies that the conditions under which pollinator shifts occur may be wider than previously thought.

In the sections below I provide background concerning these topics. I first review literature pertaining to pollinator shifts and the phenomenon and evolutionary stability of showy selfers. I then identify opportunities for expanding concepts of pollinator shifts by explicitly considering pollinator shifts in the context of theory on the maintenance of pollinator adaptations in showy selfers and describe the ecological conditions under which adaptive pollinator shifts may occur in autofertile species. Finally, I outline the studies

described in the remainder of the thesis that use *Hesperantha coccinea* to investigate these ideas.

Pollinator shifts

Reproduction of more than 80% of plant species depends on animals for pollen transport (Ollerton et al. 2011). The diverse pollinators employed include a wide range of insect species, some birds and a few other vertebrates. As a result of the contrasting morphological, sensory and behavioural characteristics of pollinators, floral traits that promote pollinator attraction and effectiveness are expected to differ among related plant species according to local pollinator assemblages (Grant and Grant 1965, Stebbins 1970). Floral variation is thought to arise from multiple processes (Johnson 2006, Strauss and Whittall 2006), including local adaptation to different pollinators associated with spatial variation in pollinator species distributions (Grant & Grant, 1965; Stebbins, 1970; e.g. Johnson, 2010; Van der Niet *et al.*, 2014). In plant species specialised for pollination by one or a few species or functionally similar groups, decreased abundance of a primary pollinator, for instance, in peripheral populations, should limit reproductive success, so that traits that promote effective pollination by a novel pollinator become advantageous in that environment (Stebbins 1970). Adaptation to different pollinators can lead to reproductive isolation (Grant 1949, Whitehead and Peakall 2014) and likely underlies much of the spectacular diversity of floral forms (Grant & Grant, 1965; Stebbins, 1970) and species richness among the angiosperms (Darwin 1877, Crepet 1984, Vamosi and Vamosi 2010).

Associations between divergence events and pollinator shifts in several lineages (Johnson et al. 1998, Beardsley et al. 2003, Whittall and Hodges 2007, Okuyama et al. 2008, reviewed in van der Niet and Johnson 2012) and the tendency of unrelated species that share the same pollinator to exhibit suites of similar traits (Faegri and van der Pijl 1979, Fenster et al. 2004) are consistent with the role of adaptation to different pollinators in floral divergence. However, such associations between pollinators and traits of fully formed species do not allow discrimination between the initial cause(s) and subsequent consequence(s) of divergence. Divergence as an active process should be studied at the level at which it occurs: between diverging populations. In this context ecotypes, populations characterized by genetically-based variation in functional traits adapted to contrasting environments, provide excellent opportunities to study the role of local adaptation to different pollinators as a driver

of floral divergence (Grant and Grant 1965). Indeed, intraspecific variation in floral traits among populations across species' ranges often parallels differences in pollination systems (e.g. Grant and Grant 1965, Armbruster 1985, Robertson and Wyatt 1990, Anderson et al. 2010, Newman et al. 2013). Among-population divergence in flower colour, odour and morphology is consistent with local adaptation due to geographic variation in pollination, ranging from qualitative variation where a novel pollinator replaces an original (van der Niet et al. 2014b) to quantitative variation such as clines in the relative abundance of pollinators (Robertson and Wyatt 1990, Valiente-Banuet et al. 2004, Dart et al. 2012) or the morphology and preference of a single pollinator (Anderson and Johnson 2009, Newman et al. 2012). Quantitative geographical variation in pollinators is particularly important for the evolution of plants with generalized pollination systems (Gómez et al. 2009, Sun et al. 2014).

Although trait-pollinator associations are consistent with local adaptation to differences in pollinator assemblages or pollinator conditioning, several other explanations, either non-adaptive or adaptive, but not pollinator mediated, are also possible. First, differences in both floral traits and pollinator fauna may simply be coincident and non-adaptive. Second, differences in pollinators among geographically separated plant morphs may reflect pollinator preference (Janzen 1985, Herrera et al. 2006, Johnson 2006), rather than differences in pollinator fauna between sites. Third, differences in floral traits, including those important for pollination, may reflect plastic responses to abiotic conditions (e.g. flower colour, Warren and Mackenzie 2001, inflorescence size, Caruso 2006, flower size and nectar volume, Halpern et al. 2010, Carroll et al. 2001). Fourth, floral traits may be directly and or indirectly subject to selection from abiotic conditions (Rausher 2008, e.g. Levin and Brack 1995, Schemske and Bierzychudek 2007) and by florivores and parasites (reviewed in Strauss and Whittall 2006). Selection by non-pollinator factors can be stronger than pollinator-mediated selection (Cariveau et al. 2004), and in some florally diverse groups pollination seems to be less involved in floral divergence than shifts in non-pollinator aspects of the environment (Goldblatt and Manning 2006, Hanley et al. 2009, Schnitzler et al. 2011). Finally, a central role of pollinators as primary drivers of floral divergence has been questioned based on observations of widespread generalization in plant-pollinator interactions, which suggest that no particular pollinator generates sufficiently strong and consistent selection to drive floral adaptation (Waser et al. 1996 Ollerton et al. 2009, but see Johnson and Steiner 2000, Fenster et al. 2004, Rosas-Guerrero et al. 2014).

Clearly, associations between pollinators and traits alone are inadequate to demonstrate pollinator-driven floral divergence (Heslop-Harrison 1958, Herrera et al. 2006).

Instead, versification of a causal role of pollinators in driving floral divergence requires specific evidence that divergence is both adaptive and pollinator driven (Rausher 2008). Local adaptation is frequently tested using reciprocal-translocation experiments (Turesson 1922, Clausen et al. 1940, reviewed by Schluter 2000, Leimu and Fischer 2008, Hargreaves et al. 2014), but this approach has rarely been applied in the context of floral adaptation to spatial variation in pollination (although see Boberg and Agren 2009, Newman et al. 2012, Sun et al. 2014). Reciprocal-translocation experiments are well suited for testing local adaptation to pollinators. Quantification of relevant fitness components, especially male success (Stanton et al. 1992, Snow and Lewis 1993, van Kleunen and Burczyk 2008), can be challenging in reciprocal translocations (Kawecki and Ebert 2004). However, seed set and pollination success can be quantified straightforwardly, enabling direct comparison of the relative contributions of alternative morphs to the next generation, as well as of the causal role of differences in pollination. Further, although local adaptation may be difficult to detect if populations are selected randomly across the range of variable species, trait-pollinator correlations can identify likely hotspots of local adaptation, because morph distribution reflects a balance of gene flow, genetic drift and local selection (sensu Gómez et al. 2009).

Although pollination success and fecundity of local forms in reciprocal translocations can provide empirical evidence that co-variation of floral traits and pollinators is adaptive and pollinator-driven, further experiments are required to identify the mechanisms of divergence. Divergence of signalling traits, such as colour and scent, among populations (Miller 1981, Anderson et al. 2010, Peter and Johnson 2014) suggests that differences in sensory perception of pollinators underlie adaptation. Choice tests can be used to establish pollinator preferences (Kelber 1997, Johnson 2000, Newman et al. 2012). Similar preferences in a range of environmental contexts provide evidence for innate, rather than conditioned preferences, of which only the former are expected to reflect patterns of repeated evolution of similar traits in unrelated species, such as among species of a pollination guild (Johnson and Bond 1994, Pauw 2006, Jürgens et al. 2013). Divergence of morphological traits, such as functional floral depth, among populations in association with differences in pollinators (Johnson and Steiner 1997, Boberg et al. 2014, van der Niet et al. 2014b) suggests that selection for mechanical fit between flowers and local pollinators underlie some cases of floral adaptation (Newman et al. 2015). The importance of mechanical fit for adaptive divergence can be evaluated by comparison of pollinator efficiency between ecotypes (cf. Miller et al. 2013). Simultaneous variation in multiple traits between ecotypes complicates identification of the specific targets of selection, but the functional significance of individual traits can be determined using

experimental manipulation (Johnson and Steiner 1997, Castellanos et al. 2004, Campbell 2009) or model flowers that differ in one trait only (Kelber 1997, Muchhala 2007, Jersakova et al. 2012), similar to the use of models in studies of the functional role of colouration in animals (Pfennig et al. 2001, Vignieri et al. 2010). Similarity of traits identified as targets of selection in other species that share the same pollinators (i.e. belong to the same pollination guild) also strongly supports the generality of a particular mechanism (Burd et al. 2014).

Showy selfers and Best-of-Both-Worlds mating

Darwin (1862) first recognized the paradox inherent in the reproductive systems of showy selfers, which are characterized by the combination of highly specialized pollination systems and mechanisms of autonomous self-fertilisation that enable reproduction independent of pollinators (Fenster and Martén-Rodríguez 2007). One explanation is that adaptations for pollinator specialization in showy selfers are retained despite no longer being functional (Barrett 2013). Alternatively, the "opposing contrivances" of showy selfers may represent an optimal "Best-of-Both-worlds" reproductive system, in which the combination of autonomous self-fertilisation and specialized pollination provides the benefits of both strategies (Darwin 1877, Kalisz and Vogler 2003, Fenster and Martén-Rodríguez 2007). Evidence that outcrossing rates vary positively with pollinator availability in variable pollination environments supports the Best-of-Both-Worlds scenario (Kalisz et al. 2004, Goodwillie and Weber 2018). However, evidence for stability of Best-of-Both-Worlds reproduction remains limited and the subject of ongoing debate, especially in relation to the stability of mixed selfing and outcrossing (Goodwillie et al. 2005, Eckert et al. 2006).

The prevalence of mixed mating in general has been considered an evolutionary enigma (Goodwillie et al. 2005). The simplest models of mating system evolution predict only two stable endpoints, predominant selfing or outcrossing, according to whether the inherent transmission advantage of self-fertilisation (Fisher 1941) is countered by reduced fitness of selfed offspring (Lloyd 1979). However, empirical evidence increasingly contradicts the predicted bimodal distribution of mating systems (Barrett and Harder 1996, Barrett and Harder 2017). Although estimates may be biased against exclusive outcrossing (Igic and Kohn 2006), broad surveys of mating system estimates based on molecular data suggest that approximately 42% of flowering plant species have intermediate outcrossing rates between 20 and 80% (Vogler and Kalisz 2001, Goodwillie et al. 2005, Whitehead et al.

2018), which seems too frequent to represent only systems in transition (Goodwillie et al. 2005). Inbreeding should evolve with selfing rates owing to purging of deleterious alleles (Lande and Schemske 1985, Charlesworth and Willis 2009), leading to a positive association between outcrossing rate and genetic load. However, similar estimates of inbreeding depression in mixed mating taxa and predominately outcrossing taxa also suggest that mixed mating represents a stable strategy rather than a system in transition to greater selfing (Winn et al. 2011). Together, the frequency of mixed mating and the lack of an association with inbreeding depression suggest that alternative explanations for stable mixed mating should be explored.

Theory that emphasizes the importance of ecological determinants of mating systems predict a wider range of conditions for stable mixed mating (e.g. Morgan and Wilson 2005, Aizen and Harder 2007, Harder et al. 2008, Johnston et al. 2009). Many of these models recognise the timing and mode of self-pollination as important determinants of selection for selfing (Lloyd 1979). Selfing that occurs simultaneously with opportunities for outcrossing carries gamete discounting costs if competing selfing usurps ovules and/or reduces the pollen available for export. In contrast, delayed selfing, which occurs only late in floral life after opportunities for outcrossing have passed, is advantageous under the widest range of conditions (Lloyd 1979). Delayed self-pollination in species with showy flowers is a key component of Best-of-Both-Worlds reproduction, as facultative autonomous self-pollination enables pollinator-mediated reproduction whenever possible while ensuring reproduction when outcrossing is limited (Becerra and Lloyd 1992, Kalisz et al. 2004, Goodwillie and Weber 2018). Models that emphasize that correlations between fertility components may also determine stable mixed mating (Johnston 1998, Johnston et al. 2009) are particularly relevant to the stability of Best-of-Both-worlds mating in showy selfers, in which relations between outcrossing and selfing rates are implicit.

How mixed mating and the contrasting contrivances of showy selfers are maintained, remains less clear. Several studies have used floral emasculations to quantify the reproductive assurance benefit of autonomous self-fertilisation (Lloyd 1992) and demonstrate the basic principle that selfing can elevate reproductive success in the face of variable pollination (Kalisz and Vogler 2003, Kalisz et al. 2004, Goodwillie and Weber 2018). Floral manipulations have similarly been used to demonstrate predominantly delayed timing of self-pollination in showy species (Leclercpotvin and Ritland 1994, Kalisz et al. 1999, Ruan et al. 2009, Elle et al. 2010, Dart and Eckert 2013b). Combined manipulative and molecular methods show the extent to which variation in selfing rates reflects response to pollinator

failure (Ruan et al. 2008, Elle et al. 2010, Koski et al. 2019). The role of inbreeding depression in maintaining intermediate selfing in Best-of-Both-Worlds systems remains ambiguous, as strong inbreeding depression limits benefits of self-fertilisation in some systems (Herlihy and Eckert 2002, Ruan et al. 2011) but not in others (Goodwillie and Weber 2018). Also, few studies have specifically addressed mechanisms of maintenance of traits for selfing and outcrossing in showy selfers, other than the influence of autofertility in reducing selection on pollinator attraction traits (Teixido and Aizen 2019). However, many studies have found associations between reliance on self-fertilisation and variation in floral traits that promote self-pollination (Goodwillie and Ness 2005, Goodwillie and Knight 2006, Kalisz et al. 2012, Torang et al. 2017) or adaptations for pollinator attraction (Goodwillie et al. 2010, Button et al. 2012). These patterns, especially those associated with geographic trends in reliance on self-fertilisation in colonized regions or range edges (Wyatt 1988, Moeller 2006, Perez et al. 2013, Bontrager et al. 2019), are more consistent with shifts to selfing in some parts of species range than with stable mixed mating (Goodwillie and Weber 2018, also see Igic and Busch 2013, Wright et al. 2013, Cheptou 2019). Therefore, the stability of mixed mating in showy selfers remains unclear.

Theoretical conditions for pollinator shifts in showy selfers

The multiple pathways by which floral traits for specialized pollination may be maintained and, by extension, enable pollinator shifts in showy selfers likely involve both female and male components of reproductive success (Fenster and Martén-Rodríguez 2007, also see Barrett and Harder 2017). Selection through female function, which is positively related to the strength of pollen limitation (Ashman and Morgan 2004), may promote adaptations for specialized pollination in showy selfers if the quantity or quality of pollen receipt limits female reproductive success (Aizen and Harder 2007). Firstly, imperfect self-compatibility or autofertility (Lloyd and Schoen 1992, Eckert et al. 2010), may constrain the effectiveness of autonomous self-fertilisation in alleviating pollen limitation. Further, inbreeding depression may limit the contributions of selfed offspring to reproductive success. Any or all of these factors may limit female reproductive success and selection favouring floral traits that promote receipt of more or better-quality pollen. Self-compatibility, autofertility and inbreeding depression vary widely among showy-selfing species and variation in inbreeding depression is particularly striking (reviewed in Goodwillie and Weber 2018). In some species

inbreeding depression strongly erodes the benefit of autonomous self-fertilisation (Herlihy and Eckert 2002, Ruan et al. 2008), whereas in others it is consistently weak during all life stages (Zink and Wheelwright 1997, Kalisz et al. 2004). Capacity for autonomous self-fertilisation reduces, but need not eliminate pollen limitation (Larson and Barrett 2000). Thus, at least in some showy selfers, selection to alleviate pollen limitation of female reproductive success may be an important mechanism maintaining adaptations for specialized pollination.

Selection for cross-promotion adaptations in showy selfers may also be maintained through variation in siring success. In particular, selection on floral traits promoting siring success increases if high self-compatibility and autofertility and weak inbreeding depression reduce variation in female success (Bell 1985, Burd 1994). This relation is consistent with an important role of male function in maintaining pollinator adaptation in showy selfers (Fenster and Martén-Rodríguez 2007, also see Paterno et al. 2020). Thus, consideration of the theoretical conditions underlying adaptive maintenance of floral traits that mediate specialized pollination in showy selfers suggests several mechanisms for pollinator shifts in autofertile species. The same selective mechanisms – limits to autofertility, self-compatibility or any degree of inbreeding depression – that mediate specialized pollination in showy selfers, in addition to the likely importance of selection though siring success, could facilitate adaptation to a novel pollinator if showy selfers undergo range expansion or otherwise encounter changes in the pollination environment.

In addition to these mechanisms by which pollinator adaptation may be maintained in Best-of-Both-Worlds systems, a recent theoretical study demonstrated that self-fertilisation may facilitate shifts between pollinators (Wessinger and Kelly 2018). Two key effects of self-fertilisation, involving reproductive assurance and genetic influences, could influence pollinator transitions in species with delayed self-fertilisation. Firstly, selfing may enable populations with specialized cross-pollination systems to persist through pollination bottleneck by buffering plants against pollinator failure. Self-fertilisation may enable shifts to less frequent, but more effective pollinators. Secondly, selfing can theoretically promote fixation of advantageous recessive loss-of-function mutations, which are commonly associated with shifts to hummingbird pollination from bee-pollinated ancestors (Wessinger and Kelly 2018). Specialized hummingbird pollination has typically evolved in association with self-compatibility, suggesting that selfing may have enabled these shifts (Wessinger and Kelly 2018). Both of these associations are consistent with Best-of-Both-Worlds mating. Thus, the possibilities that selfing may predispose pollinator shifts and that mechanisms that

promote outcrossing in showy selfers may also enable pollinator shifts merit further exploration.

Study system

Iridaceae are characterised by extensive floral diversity and reach their greatest species diversity in southern Africa (Goldblatt and Manning 2006), where a remarkable number of species are characterised by highly specialized plant-pollinator interactions (Johnson and Steiner 2003, Johnson 2010). At least seventeen pollination systems have been described in the Iridaceae, and the parallel occurrence of many pollination systems within each of the large genera suggests adaptive radiations in pollination systems (Goldblatt and Manning 2006).

Among the c. 80 species of *Hesperantha*, variation in flower colour, orientation, tube length, timing of anthesis, nectar traits and odour in association with pollination by distinct pollinator groups suggests that pollinators have been important drivers of trait divergence (Goldblatt et al. 2004). Within the genus, red flowers are unique to *Hesperantha coccinea* (Backh. and Harv.) Goldblatt and J.C.Manning, a streamside species from the summer-rainfall region of South Africa and Zimbabwe. This species is a member of a guild of other unscented, red-flowered species in diverse families pollinated by the nymphalid butterfly, *Aeropetes tulbaghia* (Linnaeus) (Johnson and Bond 1994). However, pink-flowered populations of *H. coccinea* are also known from the Drakensberg Mountain region (Goldblatt and Manning 1996b, Pooley 2003), where several other pink-flowered species with long, narrow floral tubes, dilute nectar, and an absence of floral scent are pollinated by the long-proboscid fly *Prosoeca ganglbaueri* Lichtwardt (Nemestrinidae) (Goldblatt and Manning 2000). *Hesperantha coccinea* is a valuable garden plant in Europe, where cultivated plants of *H. coccinea* set viable seed in the absence of mates and outside of the range of either pollinator species (Wolff et al. 2009). Intriguingly, the capacity for autonomous seed set has been observed for both red and pink-flowered plants (P. Goldblatt, pers. comm.). Thus, pink- and red-flowered forms of *H. coccinea* represent an ideal study system to investigate potential adaptation to different pollinators in an autofertile system.

Thesis outline

The aim of this study is to determine whether pollinator-driven adaptive divergence can occur in a clade with Best-of-Both-Worlds reproduction, using *H. coccinea* as a study system. I specifically test the hypothesis that floral variation among *H. coccinea* populations reflects adaptation to functionally distinct pollinators, and investigate whether selfing occurs in both pollination ecotypes, providing reproductive assurance, or instead have evolved as an alternative to a pollinator shift. In Chapter 2, I identify the primary pollinators, investigate covariation of floral colour, morphology and nectar characteristics and pollination across the species range and assess the genetic basis of divergent traits in a common garden. Colour convergence in flowers of *H. coccinea* and other species pollinated by *P. ganglbaueri* and *A. tulbaghia* is assessed by comparison of spectral reflectance in two vision models and choice tests are used to assess colour preferences of different pollinators. In Chapter 3, I assess evidence that floral variation reflects local adaptation to different pollinators. Comparisons of pollinator attraction and effectiveness between morphs are used to evaluate the roles of pollinator signalling and mechanical fit in floral divergence, and translocation experiments are used to evaluate local adaptation to different pollination environments. In Chapter 4, to assess the extent of reliance on pollinators for reproduction I quantify the breeding and mating systems in two populations of each colour morph. Inbreeding depression is investigated in populations of both colour morphs, from seed set to reproductive maturity (flowering) and SSR markers are used to quantify outcrossing rates. In Chapter 5, I consider evidence for Best-of-Both-Worlds reproduction in *H. coccinea*. Floral manipulations are used to determine the contributions of pollinators and autonomous self-fertilisation to reproductive success under natural pollination conditions across the species range. Relations between the extent of reliance on selfing versus pollinators and population geography and floral traits associated with autonomous self-fertilisation and specialised pollinator adaptation are investigated. In Chapter 6, I synthesize the results of the four research chapters, discuss their relevance to current understanding of intraspecific pollinator mediated divergence and Best-of-Both-Worlds reproduction and identify opportunities for future research.

CHAPTER 2: A POLLINATION GUILD SHIFT UNDERLIES FLORAL VARIATION AMONG POPULATIONS OF A SOUTH AFRICAN IRIS

Abstract

Plant adaptation to contrasting pollinators generates floral divergence within lineages and convergence among lineages, and is often represented during early stages by pollination ecotypes. Here, I consider whether pink- and red-flowered populations of the southern African iris *Hesperantha coccinea* are the products of such diversification. Spectral reflectance patterns, floral morphology and nectar traits were compared among populations under natural and common-garden conditions and with other plant species that share pollinators with these populations. Pollinator colour preferences were assessed using choice tests with model flowers. I found that flower colour of *H. coccinea* is genetically determined and associated with differences in floral morphology, orientation and nectar traits. Long-proboscid flies visited only pink models and frequented only pink-flowered populations, whereas butterflies strongly preferred red models and were the predominant pollinators in red-flowered populations. Based on general opponency and categorical fly-vision models, floral reflectance of red and pink-flowered *H. coccinea* populations is strongly convergent with that of other plants species that share pollinators with these populations. Population differences in *H. coccinea* are thus consistent with both divergent adaptive responses to the behaviour and morphology of the locally dominant pollinators and contrasting convergent evolution within their associated pollination guilds.

Key words: *Aeropetes tulbaghia*, colour preference, ecotype, *Hesperantha coccinea*, Iridaceae, long-proboscid flies, plant-pollinator interaction, *Prosoeca ganglbaueri*.

Introduction

Convergent evolution of distantly related organisms that occupy similar ecological niches is among the strongest macroevolutionary evidence for the role of adaptation in phenotypic diversification. For example, similarities in flower form, colour and scent among unrelated angiosperm species with the same pollination system are often interpreted as the result of convergent selection by pollinators (Faegri and van der Pijl 1979, Fenster et al. 2004, Rosas-Guerrero et al. 2014). Conversely, phylogenetic evidence frequently reveals floral divergence associated with shifts from one pollination system to another (van der Niet and Johnson 2012, Smith and Kriebel 2018). These two lines of macroevolutionary evidence imply widespread adaptation by plants to the local pollination environment; however, the adaptive processes underlying divergence within lineages and convergence among them necessarily act at the population level, and are therefore beyond the scope of macroevolutionary studies (Losos 2011). In contrast, consideration of inter-population variation within species, such as pollination ecotypes (Armbruster 1985, Robertson and Wyatt 1990, Johnson 1997), reveals "evolution in action" (c.f. Grant and Grant 1965) and can thus illuminate adaptive processes that generate biodiversity.

A wealth of ecotype studies now link intraspecific floral divergence with pollination differences. Many have shown evidence of co-variation between pollinator morphology and traits involved in the fit of flowers to pollinators (Armbruster 1985, Herrera et al. 2006, Nattero and Cocucci 2007), in particular, floral-tube length (Robertson and Wyatt 1990, Anderson and Johnson 2009, Anderson et al. 2010, Boberg et al. 2014, van der Niet et al. 2014b). Fewer studies have explored the role of pollinator shifts in driving population divergence in floral advertising traits, such as floral scent (Steiner et al. 2011, Sun et al. 2014, van der Niet et al. 2014b, Suinyuy and Johnson 2018) and colour (Miller 1981, Newman et al. 2012, Peterson et al. 2015). Further, although matches between flower and pollinator morphology are expected to be adaptive because of their importance for pollen exchange (Grant and Grant 1965, e.g. Cresswell 2000, Muchhala 2007, Parker et al. 2018) the effect of variation in advertisement traits on flower visitation depends on complex pollinator-specific responses, which are influenced by innate preferences, learning, and receiver bias (Schiestl and Johnson 2013). In intraspecific studies in particular, few behavioural experiments have assessed the effects of variable advertising traits on pollinator attraction (Rausher 2008, but see Newman et al. 2012, Peter and Johnson 2014).

Colour facilitates pollinator attraction (Grant and Grant 1965, Stebbins 1970) by providing a signal for the location of floral rewards that contrasts strongly with the surroundings (Schiestl and Johnson 2013). Differences among pollinators in visual systems and both innate preferences and learning ability are probably dominant influences on the evolution of the diversity of floral colour patterns (Kevan et al. 1996, Schiestl and Johnson 2013). However, changes in flower colour can also result from plastic responses to environmental differences (e.g. Stiles et al. 2007), or selection unrelated to pollinator attraction (Armbruster 2002, Rausher 2008). Thus, confirmation of pollinators as agents of flower colour divergence requires demonstration of both genetic determination of flower colour and a mechanism of pollinator-mediated selection on colour (Herrera et al. 2006). The first requirement can be demonstrated simply with common-garden experiments (e.g. Ellis and Johnson 2009). In contrast, demonstrating the importance of colour for pollinator signalling can be confounded by correlated divergence of other floral traits (Campbell 2009). This problem can be circumvented by quantifying pollinator responses to artificial or manipulated flowers that differ in only one trait (e.g. Ishii and Harder 2006; Campbell et al., 2010; Jersakova et al., 2012; Newman et al., 2012; reviewed by Campbell, 2009).

The role of pollinator preference in driving flower colour divergence is also evident in contrasting pollination guilds. Similar flower colour among unrelated plant species that share the same pollination system suggests convergent evolution resulting from pollinator preferences (Brown and Kodric-Brown 1979, Dafni et al. 1990, Helversen 1993, Burd et al. 2014). Intra-specifically, local adaptation to the colour preferences of resident pollinators should cause divergence of floral colour among populations as they converge on the colours of other plant species that share the same pollinator.

Pollinators with contrasting colour preferences also likely differ in other morphological and behavioural traits that could affect pollination. Therefore, attraction of functionally different pollinators should alter selection on other floral traits (Stebbins 1970), including reward characteristics (e.g. Dupont et al. 2004, Kromer et al. 2008, Parker et al. 2018) and morphological traits mediating pollinator behaviour (Castellanos et al. 2004) and fit (Anderson and Johnson 2009). Thus, a pollinator shift involving modification of flower colour should be accompanied by divergence in a suite of floral traits.

The flora of southern Africa exhibits exceptional floral diversity and endemism (Goldblatt and Manning 2002b) making it ideal for studying pollinator-mediated diversification. In particular, several groups of specialized flower visitors, such as large butterflies (Johnson and Bond 1994), long-proboscid flies (Manning and Goldblatt 1996,

1997) and oil-collecting bees (Pauw 2006) represent unique ecological niche axes that promote convergent adaptation in their associated plant guilds (Johnson 2010). Within the southern African flora, most of the > 1000 species of Iridaceae (Goldblatt and Manning 2006) depend on a single pollinator or pollinator functional group (Johnson and Steiner 2003). Importantly, diversification in this family is associated with shifts between specialised pollination systems and accompanying modifications of floral traits (Goldblatt et al. 1995, Goldblatt et al. 2000, Goldblatt and Manning 2007). Despite this abundant macroevolutionary evidence for the role of pollinator shifts in species divergence, demonstration of the microevolutionary processes underlying floral divergence in the Iridaceae is surprisingly limited (but see Anderson et al. 2010, Newman et al. 2013).

I investigated whether floral variation among populations of *Hesperantha coccinea* (Backh. and Harv.) Goldblatt and J.C.Manning, a southern African iris, is consistent with adaptation to functionally distinct pollinators with contrasting colour preferences. In the genus *Hesperantha*, the floral bauplan is conserved, but shifts in traits including tepal colour and orientation, floral-tube length and scent are associated with contrasting pollination systems (Goldblatt et al. 2004). Within the genus, red flowers are unique to *H. coccinea* (Fig. 2.1 a) a streamside species that is a member of a guild of other unscented, red-flowered species pollinated by the nymphalid butterfly, *Aeropetes tulbaghia* (Linnaeus), which settles while feeding (Fig. 2.1 b, Johnson & Bond, 1994). However, pink-flowered populations of *H. coccinea* are also known from the Drakensberg Mountain region (Goldblatt and Manning 1996b, Pooley 2003), particularly at higher altitudes where several other pink-flowered plant species with long, narrow floral tubes, dilute nectar, and an absence of floral scent are pollinated by the long-proboscid fly *Prosoeca ganglbaueri* Lichtwardt (Nemestrinidae), which hovers while feeding (Fig. 2.1 c, d; Goldblatt and Manning 2000). I therefore hypothesised that contrasting pollinator preferences drive colour variation among *H. coccinea* populations, causing intraspecific trait divergence associated with occupation of two distinct pollination niches (butterflies and long-proboscid flies).

I assessed this hypothesis by testing a set of associated predictions. (1) Flower-colour variation in *H. coccinea* is genetically determined. (2) Red- and pink-flowered populations are pollinated by butterflies and long-proboscid flies, respectively, which (3) differ in their geographic distribution and (4) have contrasting preferences for red and pink. (5) Flower colour co-varies among populations with other floral morphological traits that facilitate pollination by the respective pollinators. (6) Spectral reflectance of *H. coccinea* flowers from

different populations is convergent with that of other plant species that share the same pollinators.

Materials and Methods

Study species and sites

Hesperantha coccinea is a perennial geophyte that grows along montane watercourses (Fig. 2.1 a) from the Drakensberg escarpment in the Eastern Cape of South Africa to the highlands of eastern Zimbabwe (Goldblatt and Manning 1996b). Study sites were selected to represent flower colour variation throughout the range of *H. coccinea*, based on field observations and herbarium records (PRE, NBG, BOL, GRA and NU). Fieldwork was conducted at 50 sites between January and April of 2009-2014. Table A1 in the Appendix presents details of the study sites

Floral traits

For each *H. coccinea* population I estimated the mean floral reflectance spectrum based on a median of 8 flowers, each sampled from a different plant to ensure independent sampling (see Table A2.1 in Appendix for all sample sizes). Spectral reflectance from 300-700 nm was measured from the centre of the upper surface of one outer tepal per flower using an Ocean Optics (Dunedin, FL, USA) USB 4500 spectrometer. The fibre optic reflection probe (QR-400-7-UV-VIS; 400 μm) was held 5 mm from the tepal surface and was angled parallel with the longitudinal axis of each tepal. Preliminary measurements showed negligible spectral variation across the tepal surface (data not shown).

The primary spectral difference between pink and red is the extent to which violet light (380-450 nm) is reflected: populations with floral spectra with maximal mean violet reflectance <10% were classified as 'red', whereas those with maximal mean reflectance >20% were classified as 'light pink', corresponding to human perception of these spectra. Populations with maxim mean violet reflectance of 10-20% were classified as 'dark pink'. Four of the 50 populations (Tugela, White Mountain, Giants Castle and Nkolweni), comprised mixtures of light pink and red-flowered plants. These "mixed" populations were excluded from statistical analyses for which population colour was treated as a categorical predictor variable.

To map colour variation over the geographical range of *H. coccinea*, I used herbarium specimens for some populations that I could not access. In these cases, collector records concerning flower colour were categorised as follows: "vermilion", "scarlet", and "crimson" were considered red; whereas "madder lake", "salmon" and "pale pink" were considered pink. To investigate elevational variation of flower colour, I plotted the frequency distributions of sampled populations and localities of herbarium specimens with red and pink-flowered plants against elevation.

I investigated whether flower morphology, orientation, nectar traits and display size vary among *H. coccinea* populations and correspond with differences in flower colour. Floral dimensions were measured to the nearest 0.1 mm using digital callipers. Tube length was measured from the top of the ovary to the mouth of the floral tube. For one outer tepal per flower, I measured the free length beyond the mouth of the floral tube and the maximum width. Anther and stigma exertion were measured from the mouth of the floral tube to the apex for one randomly selected anther or stigma branch per flower. Floral orientation (angle between the plane of the flower face and the horizontal plane) was measured using a Wixey WR300 digital protractor (Barry Wixey Development, Seattle, Wash. USA). The numbers of open flowers (display size) and total flowers (including wilted flowers and buds), and plant height (a measure of plant size) from the base of the sheathing leaf to the inflorescence tip were also recorded for one inflorescence per plant.

Nectar characteristics were measured from cut exposed flowers in the field or cut inflorescences on the collection day (see Herrera, Perez & Alonso, 2006b). The ovary was separated from the base of the floral tube and nectar was extracted by capillary action into calibrated micropipettes to measure nectar volume (Fisherbrand 1-5 µl). Nectar concentration was measured using a Bellingham and Stanley 0-50% pocket refractometer. Nectar composition was quantified using high performance liquid chromatography (HPLC) according to the methods described by van der Niet et al. (2010).

I assessed whether morphological and nectar traits differed among light pink, dark pink and red populations with generalised linear models (GLM). To account for repeated measurement of individual populations for all traits, except nectar concentration and composition, these analyses used generalised estimating equations (Liang & Zeger, 1986) with an exchangeable correlation matrix, as implemented in SPSS 21 (IBM Inc.). Analyses of plant height, nectar volume and floral dimensions considered Gaussian distributions and identity link functions, whereas those of flower numbers considered the Poisson distribution and log link function. Associated inference involved score (T) tests, which follow the χ^2

distribution (Boos, 1992). The analyses of nectar concentration and sucrose proportions considered beta distributions as implemented in SAS 9.4 (glimmix procedure; SAS Institute Inc., 2013). These analyses involved generalised linear mixed models (GLMM) that used the sandwich estimator of the covariance matrix of flower colour and implemented robust Wald hypothesis tests (Fay & Graubard, 2001), which follow the *F* distribution. In all cases, the Dunn-Šidák procedure was used for *a posteriori* pairwise comparisons among means for populations with red, light pink and dark pink flowers. Numbers of populations and flowers sampled for each trait are given in Table 2.1.

Genetic determination of flower colour

To establish whether variation in flower colour and morphological traits has a genetic basis, traits were compared between representative populations *in situ* and grown from seed in a common garden. The latter plants were derived from wild-collected seeds and grown at the Botanical Gardens of the University of KwaZulu-Natal (Pietermaritzburg campus). Individual fruits were collected from a minimum of 20 plants separated by ≥ 5 m in two red (Bushman's Nek and Elliot), one dark pink (Kamberg) and three light pink-flowered (Devil's Hoek, Golden Gate and Mahai) populations of *H. coccinea*. During autumn, seeds were sown into 27-cm diameter pots with a 3:1 mixture of potting soil to river sand. Pots were initially kept in a glasshouse to reduce the effects of low overnight temperatures on germination. After one year, the established plants were moved outside to a shadehouse. Inflorescences developed mostly during the second year after sowing. The reflectance spectrum of one flower per inflorescence from up to three individuals per seed family was measured spectrophotometrically and then converted to a locus in Endler's (1990) colour space. In this space, hue (colour as perceived by humans, e.g. red, blue, green) is represented by the angle between the y-axis and the spectral point, and chroma, which measures colour saturation (pale to intense), is represented by the point's distance from the origin. Position along the y-axis is determined by the difference in summed reflectance between the 300 to 400nm region (UV to violet wavelengths) and the 600 to 700 nm region (red wavelengths), whereas position on the x-axis is determined by difference in summed reflectance between the 400 to 500 nm region (blue-green wavelengths) and the 500 to 600 nm region (yellow to orange wavelengths). Euclidean distance between points in this colour space is proportional to spectral similarity. To establish whether variation in floral morphological traits has a genetic

basis, I also measured a representative subset of morphological traits for flowers of the two red and three light pink populations.

I assessed the genetic determination of traits in two ways. First, I used reduced major axis regressions to test whether trait means of *in situ* and garden-grown plants from each population varied positively. Significance of regression relations was assessed based on the probability of the observed *F*-ratio arising from sampling error alone, as determined from 9999 permutations of group membership as implemented in the program PAST (Hammer, Harper & Ryan, 2001). Second, genetic and environmental components of variation in flower colour were approximated as the variance attributable to source population and environment (*in situ* vs. common garden), respectively, in a non-parametric multivariate analysis of chroma and hue using a two-factor permutation analysis of variance. Pairwise post-hoc comparisons used similar permutation tests with the Dunn-Šidák adjustment of the Type I error rate for individual comparisons.

Pollinator distributions, observations and colour choice

Pollinator distributions were mapped based on collection localities of specimens from the South African Butterfly Conservation Assessment (2009) (*A. tulbaghia*) and Goldblatt and Manning (2000) (*P. ganglbaueri*). To determine the elevation range of each species, I examined databased collections of the Iziko South African Museum (SAM), Albany Museum at Grahamstown (AMG), KwaZulu-Natal Museum at Pietermaritzburg (NMSA) and South African Butterfly Atlas Project (Mecenero et al. 2013) and augmented these with my own collections housed at the University of KwaZulu-Natal.

To determine whether pollinators differed among *H. coccinea* populations according to flower colour, floral visitors were observed during sunny conditions for an average of 19 h (total 285 h, range 6-44 h) in each of 15 populations that were selected to encompass the range of flower colour variation. Flowers of *H. coccinea* close overnight (Goldblatt *et al.*, 2004; R.J. Cozien, pers. obs.), so nocturnal observations were not needed. All insects that contacted both anthers and stigma branches were counted and identified by comparison with previously captured and identified individuals. To establish whether different visitor species pollinated flowers, I assessed pollen deposition after single visits to emasculated virgin flowers (Primack and Silander 1975). Visited stigmas were mounted in fuchsin gel to stain pollen grains (Beattie 1971) and the pollen grains were counted at 80× magnification. Pollen deposition was compared among visitor species using a GLM with a negative binomial

distribution and log link function in SPSS 21 (IBM Inc.). The Dunn-Šidák procedure was used for *a posteriori* pairwise comparisons among means.

To determine whether *Aeropetes tulbaghia* and *Prosoeca ganglbaueri*, the primary pollinators in red- and pink-flowered populations, respectively (see Results), exhibit contrasting colour preferences, I offered artificial red and pink model flowers to them at four sites (Witzieshoek, Mahai, Golden Gate, Bushman's Nek). Artificial flowers were constructed from clear acetate plastic (overhead transparency) which had been painted with acrylic paint ("barney purple" and "berry red 10", Scrapbook Creations, Hayfields Mall, Pietermaritzburg, South Africa: see Jersakova et al. (2012) for details and images of these flowers). Colour spectra of these artificial flowers were very similar to those of red and light pink *H. coccinea* (Fig. A2.1 in Appendix). During each trial, 8 flowers of a given colour were arranged in a 15-cm raceme. Two inflorescences, one of each colour, separated by 40 cm were presented to insects using Thomson's (1988) presentation-stick method, as modified by Johnson (2000). In total, 61 trials were conducted during nine days at the four sites.

For each insect that approached the presented inflorescences, I recorded the flower colour that it first examined or probed. To assess whether butterflies and flies exhibited contrasting colour preferences, I compared the proportion of first approaches to pink flowers between flies and butterflies with likelihood-ratio (G) tests in GLMs that considered binomial distributions. I also assessed whether each insect type exhibited significant preference for a particular colour by testing whether the mean logit differed significantly from 0 (equivalent to a proportion of 0.5 or equal choice). Analyses were implemented in SPSS 21 (IBM Inc.). For illustrations, means and confidence intervals were back-transformed from the logit scale used for analysis.

Flower colour in pollination guilds

To assess the correspondence of flower colour in *H. coccinea* populations to the general floral colour phenotypes of other species pollinated by *A. tulbaghia* and *P. ganglbaueri*, I surveyed floral spectral reflectance of species within both pollination guilds. For the *A. tulbaghia* guild I used spectra for eight species reported by Johnson and Bond (1994) plus spectra that I measured as described above for three additional species sampled at our study sites (see Fig. A2.1 and Table A2.2 in Appendix). I also sampled spectra for 16 species in the *P. ganglbaueri* pollination guild (Goldblatt and Manning, 2000; Anderson and Johnson, 2009; personal observations: see Fig. A2.1 in Appendix). Colour spectra were collected from

three to 12 flowers from one to three populations of each guild member (median sample = 8 flowers, for details see Table A2.2). I measured the colour of the floral part 'facing' the approaching pollinator. For a reference spectrum of the visual background, spectra of eight *H. coccinea* leaves were measured.

The visual systems of *A. tulbaghia* or *P. ganglbaueri* are unknown, complicating spectral comparison from the pollinators' perspectives. Butterflies have exceptionally diverse visual systems (Osorio & Vorobyev, 2008), so the system used by *A. tulbaghia* is difficult to predict. I therefore applied Endler's (1990) model to represent butterfly vision, as it is relatively robust to differences in spectral sensitivities particular to any vision system (Endler, 1990). Chroma and hue of *H. coccinea* populations and species of the respective pollination guilds were compared first with a two-factor permutation multivariate ANOVA and then individually with similar, single-factor analyses using the program PAST (Hammer *et al.*, 2001). In all analyses, the Dunn-Šidák procedure was used for post-hoc pairwise comparisons among means.

Endler's (1990) model cannot be used for flies, as their colour vision differs fundamentally from other known systems in that colour discrimination is believed to be categorical, rather than continuous (Troje 1993). Therefore, to compare colours of *H. coccinea* flowers and guild members as perceived by flies, I plotted spectra in the colour space of Troje's (1993) model of fly vision. This model was devised for flower-feeding *Lucilia* flies, using spectral sensitivities of *Musca* and *Lucilia* (Hardie & Kirschfeld, 1983), but it approximates vision for pollinating flies in general (Arnold, Savolainen & Chittka, 2009) and nectar-feeding tabanids in particular (Jersakova *et al.*, 2012), and is thus a reasonable starting point for interpreting colour from the perspective of nemestrinid flies. In this model, spectral stimuli are distinguished among, but not within, three wavelength ranges, irrespective of the magnitude of the difference between them. Category transitions occur at approximately 400 and 515 nm. The system is based on two pairs ("y", yellow and "p", pale) of antagonistic receptors. Depending on which receptor of each pair is stimulated more strongly, four fly colours are perceived: fly UV, p+ y+; fly blue p- y+; fly yellow p- y- and fly purple p+ y- (Troje, 1993). According to the model, flies distinguish colour stimuli that lie in different quadrants, but distance between loci in colour space otherwise conveys no information.

Results

Geographical distribution of flower colour and floral variation

Variation in flower colour among *H. coccinea* populations is structured both latitudinally and elevationally (Fig. 2.2). As reflected in its specific epithet, most *H. coccinea* populations are red-flowered (hereafter red populations). Populations with dark pink flowers (dark pink populations) occur mainly in the central Drakensberg, except for a small cluster of populations in the north-east of the range, along the Mpumalanga escarpment. Populations with light pink flowers (light pink populations) are concentrated in the centre of the species' range in the northern Drakensberg, mostly northwest of dark pink populations. In this region, populations with light pink, dark pink or red flowers are located in close proximity, even within the same river basin. The four mixed populations occur in this area. With respect to elevation, red-flowered populations occurred almost throughout the elevation range of *H. coccinea* from 800 m.a.s.l to 1900 m.a.s.l. All populations between 800 and 1200 m.a.s.l. had red-flowered plants and 90% of red-flowered populations occurred in the lower half of the species' elevation range. In contrast, 60% of pink-flowered populations occurred in the upper half of the species elevation range (Fig 2.3).

Most measured floral traits varied significantly among populations in association with flower colour (Table 2.1). Compared to flowers in light pink populations, those in red populations had wider tepals and shorter stamens, stigma branches and floral tubes, and faced upward. In contrast, free-tepal length, a measure of overall flower size, did not differ significantly between population types. Except for tepal width, morphological traits in dark pink populations resembled those in light pink populations. Nectar concentration and volume were similar among population types; however, nectar in red populations had much lower sucrose content (and thus higher hexose content) than that in pink populations (Table 2.1).

Genetic basis of floral variation

Flowers of plants grown from seed in a common garden produced flowers with spectral reflectance patterns almost identical to those of the parental populations in their natural environments (Fig. 2.4 a-c). More than 90% of the variation in mean floral hue and chroma in the common garden was attributed to variation among source populations (see Fig. 2.4 d, e). Correspondingly, multivariate analysis of chroma and hue showed that flower colour differed significantly among source populations ($F_{5,205} = 59.9$, $P < 0.001$), but not between growth

environments ($F_{1,205} = 0.0004$, $P > 0.9$). Thus, flower colour variation has a strong genetic basis.

As for floral colour, *in situ* differences among populations in other floral traits were maintained by seed-derived plants cultivated in the common garden (Table 2.2). For floral tube length, tepal width and sucrose composition, more than 90% of variation in the common garden was associated with differences among populations ($r^2 > 0.9$, $P < 0.01$ for all cases; see Fig. 2.5). In contrast, mean flower orientation in the common garden varied independently of mean orientation *in situ* (Fig 2.5). Thus, most inter-population associations of floral morphology with flower colour seem to be genetically based for most traits.

Pollinator effectiveness, distribution and colour preferences

Both *A. tulbaghia* and *P. ganglbaueri* deposited a mean of c. 90 pollen grains on stigmas during individual flower visits (Fig. 2.6), with no significant difference between them ($G_1 = 0.58$, $P > 0.4$); hence, they are considered equally effective pollinators. In contrast, a day-flying hawk moth, *Macroglossum trochilus* that visited flowers in most light pink populations (Table 2.3) rarely deposited pollen (Fig. 2.6), and so acted primarily as a nectar thief.

Differences in flower colour among *H. coccinea* populations were associated strongly with the dominant pollinator species. In four of the five red populations, *A. tulbaghia* was the only pollinator observed (Fig. 2.6 and Fig, 2.7), whereas at the fifth, lowest elevation site (Karkloof), two other butterfly species, *Papilio ophidiocephalus* and *P. nireus*, were observed. A greater diversity of pollinators was active in pink-flowered populations. At Dullstroom and Kamberg (both dark pink) and at Golden Gate (light pink), *P. ganglbaueri* was the dominant pollinator (Fig. 2.1 and Fig. 2.7), and it also pollinated flowers at Mahai (light pink).. This fly was not observed visiting flowers in any red-flowered population. In four of the five populations with light pink flowers, *A. tulbaghia* also visited flowers; however, it was not the most frequent pollinator in any of these populations. Additionally, the likelihood that an *A. tulbaghia* individual on the wing in the vicinity of a *H. coccinea* population was observed visiting *H. coccinea* flowers was three times higher in red-flowered populations (72% of 179 individuals observed) than in pink-flowered populations (24% of 110 individuals, $\chi_1^2 = 21.69$, $P < 0.001$; Table 2.3). Plants in mixed populations received visits primarily from the butterflies, *Papilio nireus* and *P. demodocus* (Fig. 2.7). In the mixed population at Giant's Castle I observed a single visit by *A. tulbaghia*, but neither *P. ganglbaueri* nor *M. trochilus* were observed in mixed populations.

The distributions of *A. tulbaghia* and *P. ganglbaueri*, the primary pollinators of *H. coccinea,* are largely coincident with that of *H. coccinea* (Fig. 2.1). *Prosoeca ganglbaueri* has not been recorded from a small area in the north-west of the range of *H. coccinea*, but there is otherwise little latitudinal structuring in overall pollinator distributions (Fig. 2.1). However, *A. tulbaghia* appears to occur over a wider elevation range than *P. ganglbaueri*. Databased collections of butterflies include localities from 100 m above sea level to mountain areas at 2800 m.a.s.l., whereas records of *P. ganglbaueri* suggest that the species generally occurs at high elevations, including above 3000 m.a.s.l., but not below 1400 m.a.s.l. (Fig. 2.7).

Prosoeca ganglbaueri and *A. tulbaghia* exhibited strongly contrasting preferences when confronted with a choice between pink and red model flowers ($G_1 = 27.62$, $P < 0.001$; Fig. 2.8). All *P. ganglbaueri* individuals chose pink during their first approach and during all subsequent approaches, and almost all (25 of 27) *A. tulbaghia* individuals chose red during their first approaches and did not return for subsequent approaches.

Flower colour in pollination guilds

Plant species in both the *P. ganglbaueri* and *A. tulbaghia* pollination guilds exhibit floral spectral reflectance with maximum reflectance at approximately 620 nm (red) (Fig. A2.1 in Appendix), but they differ in the presence of a secondary peak between 400 – 450 nm. For species belonging to the *P. ganglbaueri* guild, reflectance in the latter wavelength range varies between 20 and 40%, whereas it is absent or less than 10 % in species pollinated by *A. tulbaghia* (Fig. A2.1 in Appendix).

Based on Endler's segment classification, spectral loci of species belonging the two pollination guilds formed two distinct clusters, separated by both chroma and hue (Fig. 2.9). Red-flowered *H. coccinea* lie within the colour space of other species pollinated by *A. tulbaghia*, whereas pink-flowered plants lie within the colour space of species pollinated by *P. ganglbaueri*. In general, dark pink populations were closer to the colour phenotype space of species pollinated by *A. tulbaghia* than of those pollinated by *P. ganglbaueri.* Results of statistical analyses are consistent with this interpretation of colour space. Multivariate analysis considering both chroma and hue detected significant flower colour differences among pollination guilds and *H. coccinea* populations ($F_{4,69} = 63.5$, $P < 0.001$). In post-hoc comparisons, flower colour of species of the *P. ganglbaueri* guild did not differ from that of light pink-flowered *H. coccinea* ($F = 1.19$, $P > 0.2$), and flower colour of species in the

butterfly-pollinated guild did not differ from either red or dark pink-flowered populations of *H. coccinea* ($F = 2.83$, $P = 0.78$; $F = 4.29$, $P = 0.47$). In contrast, all other groups differed significantly (F =16 to $F = 52$, all corrected $P = 0.001$). The analysis of hue alone detected the same pattern as was found for colour. However, when chroma alone was considered, all groups differed, except the *A. tulbaghia* pollination guild and red-flowered populations of *H. coccinea* (hue, overall $F_{4,69} = 25.6$, $P < 0.0001$; post-hoc differences $F = 16.2$ to $F = 52.3$, all $P \leq 0.001$ except *Prosoeca* guild vs. light pink $F = 1.19$, $P = 0.28$, *Aeropetes* guild vs. red $F = 2.83$, $P = 0.08$ and dark pink $F = 4.29$, $P = 0.04$; chroma, overall $F_{4,69} = 176.7$, $P < 0.0001$; post-hoc differences $F = 20.6$ to $F = 673.7$, all $P < 0.001$ except red vs. *Aeropetes* guild, $F = 0.04$, $P = 0.84$).

Based on the fly vision model, flower spectra for almost all samples lie in two of the four possible quadrants (Fig. 2.9a). Spectra of all red-flowered populations of *H. coccinea* lie in the "fly-UV" quadrant, along with other guild members pollinated by *A. tulbaghia* (Fig. 2.9b). By contrast, spectra of all light pink populations of *H. coccinea*, and most of the dark pink populations lie in the "fly-blue" spectral space of guild members pollinated by *P. ganglbaueri*.

Discussion

Results of this study are consistent with population divergence in floral traits of *Hesperantha coccinea* caused by a pollinator shift. Red-flowered populations are strongly associated with pollination by the butterfly *Aeropetes tulbaghia,* whereas pink-flowered populations are instead associated with pollination by the long-proboscid fly *Prosoeca ganglbaueri* (Fig. 2.1 and 2.7). Single-visit pollen deposition experiments confirmed that both species effectively pollinate flowers of the ecotype with which they associate and thus are potential agents of selection (Fig. 2.6). Furthermore, these insects have strongly contrasting colour preferences (Fig. 2.8), such that consistent, disproportionate visitation by one of them, due to a factor such as a change in local abundance of the insect or colonization of a new site or change in elevation by the plants, would promote pollination of plants with their preferred flower colour. Such phenotypic selection should promote floral evolution in populations, because flower colour differences are genetically determined (Fig. 2.4). Indeed, co-variation between flower colour, flower form, orientation and nectar composition (Tables 2.1 and 2.2) suggests that additional pollinator-mediated selection on rewards and pollinator fit accompanied

selection on floral signals, generating pollination ecotypes. It is clear that the intraspecific divergence of flower colour (and presumably other traits) did not occur randomly, as the flower-colour differences among *H. coccinea* populations align closely with the discrete phenotype spaces that characterize the guilds of other species pollinated respectively by *A. tulbaghia* and *P. ganglbaueri* (Fig. 2.9). This contrasting convergence provides additional evidence for pollinator-driven floral adaptation.

Flower colour and intra-specific divergence

Before considering the adaptive scenario outlined above, I address whether the differences in flower colour among *H. coccinea* ecotypes could instead reflect selection by agents other than pollinators. Such selection has been implicated in other cases of flower-colour divergence (reviewed in Strauss and Whittall 2006, Rausher 2008), in particular that of anthocyanin-based red and pink pigments (Warren and Mackenzie 2001). Moisture stress is the most commonly identified non-pollinator cause of anthocyanin-related colour divergence (Strauss and Whittall, 2006), but it is unlikely to explain colour differences among *H. coccinea* populations, as they all occur along perennial streams, with plants often rooted below the water line. Further, leaf anthocyanin content in *H. coccinea* is unrelated to flower colour (R.J. Cozien, unpublished results). Thus, colour divergence in response to selection on anthocyanin for vegetative function (c.f. Warren & Mackenzie, 2001) is unlikely.

Instead, several lines of evidence implicate pollinators in flower colour divergence in *H. coccinea*. Most compelling are the almost strict preference of free-foraging *A. tulbaghia* for red and of *P. ganglbaueri* for pink when confronted with a choice of artificial flowers (Fig. 2.8), and the convergence of the contrasting flower colours with those of other plant species comprising the respective pollination guilds of the two primary pollinators (Fig. 2.9). Thus, flower colour appears to be a target of selection, rather than a by-product (Chittka and Menzel 1992, Dyer et al. 2012, Shrestha et al. 2013). Because of the remarkable convergence in flower colour among guild members, the colour preferences of the two main pollinators are likely innate, but a role for learning through experience with local nectar sources (cf. Newman et al. 2012) cannot be excluded. Regardless of whether colour preference is learned or innate, that the colour morphs represent the outcome of selection by pollinators seems highly likely based on the results of the evidence presented here and the results of reciprocal transplants testing whether colour morphs have higher pollinator-mediated fitness in their local environments (Chapter 3).

Geographic basis of intra-specific divergence

Novel environmental conditions that drive adaptive differentiation are particularly likely towards the periphery of a species' range (Turesson 1922), so that pollination ecotypes often evolve where species ranges extend beyond the limits of an original pollinator (Grant and Grant 1965, e.g. Johnson and Steiner 1997, van der Niet et al. 2010, Newman et al. 2012, Cosacov et al. 2014, van der Niet et al. 2014b) Although the geographic ranges of *P. ganglbaueri* and *A. tulbaghia* largely coincide (Fig 2.2), underlying differences in their elevational distributions may promote ecotypic divergence among *H. coccinea* populations. *Aeropetes tulbaghia* occurs from sea level to montane elevations, throughout the elevation range of *H. coccinea*, whereas *P. ganglbaueri* is restricted to higher elevations (Fig 2.3). Flower colour varies similarly among populations: red-flowered populations occur throughout the elevation range of *H. coccinea*, but with pink-flowered populations predominate at high elevation and are absent at the lowest elevations (Fig. 2.3). This pattern suggests that adaptations for butterfly pollination may have followed colonization of lower elevation sites and regions where flies were absent, potentially facilitated by downstream dispersal via hydrochorous seeds (Goldblatt and Manning 1996b). Evidence consistent with adaptation for pollination by flies in montane populations has been found in other species (Sun et al. 2014) and, in general, flies are often more predominant and more important pollinators in communities at higher elevations (Arroyo et al. 1985, Kearns 1992, Totland 1992, Gray et al. 2018). In *H. coccinea*, red flowers at high elevation may represent subsequent upstream colonization, or local adaptation at high elevations to butterfly pollination. The few populations with pink-flowered plants at lower elevations may be the result of downstream seed flow, or may be pollinated by butterflies, which occasionally visit pink flowers. If so, these populations may represent progenitors for ecotype transitions.

Pollination ecotypes are proposed to evolve along lines of genetic least resistance via an intermediate stage of double function (Stebbins 1970, also see Armbruster 1993, Schluter 1996). Intriguingly, vision differences between the dominant pollinators may enable the transitional stage of double function in *H. coccinea*. As perceived by categorical fly vision, dark pink (intermediate) flowers resemble pink flowers of other species pollinated by long-proboscid flies (Fig. 2.9); whereas with any non-categorical colour vision system that perceives colour gradients, the same flowers are likely more similar to red. This interpretation is consistent with observations that pollinators of both guilds visit pink-flowered *H. coccinea*

(Fig. 2.7), which should enable a pollinator shift in either direction if the relative abundance of *A. tulbaghia* and *P. ganglbaueri* changes. Transition between long-proboscid fly and butterfly pollination along lines of least resistance (Stebbins 1970, Schluter 1996) may be further facilitated by shared key traits, including vivid flower colours, a narrow, elongated floral tube with nectar at the base, absence of floral scent, and late-summer flowering (cf. Goldblatt and Manning, 2006; see also Vogel 1954). Given that pink model flowers readily attracted flies, whereas identically shaped red flowers attracted butterflies (Fig. 2.8), a change as simple as an increase in anthocyanin concentration could initiate this pollinator shift (cf. Bradshaw and Schemske 2003). Although intermediate flower colour (dark pink in this case), could represent a stage of double function, the two primary pollinators were notably rare in the mixed populations suggesting that there may be a trade-off in attraction such that intermediate forms are less successful than either light pink or red forms. This could explain why hue is bimodal in this system with peaks corresponding to red and light pink.

Direction of the pollinator shift(s)

Within *Hesperantha*, red flowers and butterfly pollination are unique to *H. coccinea*, whereas several other species have pink flowers and are fly-pollinated (Goldblatt *et al.*, 2004). This pattern is consistent with red flowers being derived in the genus, and in *H. coccinea* in particular. The history of the disjunct distribution of pink-flowered populations in two regions, both surrounded by red-flowered populations (Fig. 2.2), is less clear, as no evidence uniquely supports parallel shifts to red, or parallel reversions to pink. Resolution of the number and direction of shifts underlying the current distribution of floral variation in *H. coccinea* will benefit from the application of genetic techniques (van der Niet et al. 2014b).

Associated floral divergence

I have proposed that differences in intrinsic colour preferences between long-proboscid flies and butterflies were key to the shift in pollination systems, but other traits such as floral morphology and nectar composition also appear to have evolved as part of this transition. Although I did not specifically test the functions of floral traits of *H. coccinea* other than colour, the morphological traits that covary with colour and pollinator type (Table 2.1) were likely also targets of selection by pollinators. The greater functional floral depth (tube, stamen and style length) in pink flowers is consistent with a positive correlation between flower depth and reproductive success in species pollinated by hovering long-proboscid flies

(Johnson and Steiner 1997, Anderson and Johnson 2009, Pauw et al. 2009). Butterflies, by contrast, probably impose less selection on flower depth, as they can adjust their probing to various flower depths while feeding in a settled position (Krenn 1990, 2010). Although proboscis lengths of both pollinators vary among sites, population means reach 55 mm in *P. ganglbaueri*, but are not known to exceed 35 mm in *A. tulbaghia* (Johnson, 2010; R.J. Cozien, unpublished). Wider tepals and upward facing flowers, both well developed in red-flowered populations, provide a landing platform for settling butterfly pollinators (Faegri and van der Pijl 1979) and facilitate pollen deposition on butterfly wings (Butler and Johnson 2020). In contrast, more dissected outlines, such as those provided by the narrower tepals of pink-flowers, are known to be more attractive to long-proboscid flies, which hover while feeding (Jersakova et al. 2012). The more lateral orientation of pink flowers probably promotes more effective pollination by their hovering pollinators, as it does for hummingbird-pollinated plants (Fenster et al. 2009, Sapir and Dudley 2013). Finally, in section Crocoideae of the Iridaceae, which includes *Hesperantha*, higher hexose nectar composition occurs more frequently in butterfly-pollinated species than in fly-pollinated species (Goldblatt and Manning 2002a, 2006). The higher energy concentration of sucrose relative to hexose may also facilitate energetically intensive hovering, as has been suggested for other pollinators (Baker and Baker 1983). Within this context, *H. coccinea* is intriguing, as it is the first example, to our knowledge, of intraspecific variation in nectar-sugar composition associated with a pollinator shift. No differences in nectar volume or concentration were detected between colour morphs; however, this could be an artefact of sampling design, which did not account for potential effects of recent environmental conditions or differences in visitation (Willmer 2011).

Biotic versus abiotic drivers of divergence

Although the Iridaceae are characterised by the extensive variation in floral traits and diverse pollination systems that are frequently associated with speciation events (Goldblatt and Manning 2006, Valente et al. 2012), the role of pollinators in divergence within this family has been downplayed compared to other ecological influences, such as soil differences (Goldblatt and Manning 1996a, Schnitzler et al. 2011, but see Forest et al. 2014). In contrast, flower traits of *H. coccinea* have apparently diverged in direct response to selection by pollinators, without any obvious shift in the habitat or vegetative traits among populations.

Thus, pollinator-driven diversification can occur in the Iridaceae in the absence of other aspects of ecological divergence.

Correspondence of micro- and macroevolutionary patterns

Pollinators are credited with driving divergence in florally diverse groups, but most current evidence comes from either studies of selection within populations (reviewed in Harder and Johnson 2009), of closely related species (Meléndez-Ackerman and Campbell 1998, Fulton and Hodges 1999, Bradshaw and Schemske 2003), or of macroevolutionary patterns (Kay and Sargent 2009, van der Niet and Johnson 2012, Smith and Kriebel 2018) Ecotypes are informative in this context, because they represent an intermediate stage of divergence during which adaptive traits have spread beyond an original population, but they have yet to become fixed at the species level. In this study, the microevolutionary pattern of divergence in flower colour, morphology and nectar composition corresponds with the macroevolutionary patterns in the pollination guilds associated with long-proboscid flies and butterflies. Thus, pollinator shifts initiated by contrasting colour preferences between these pollinators and reinforced by subsequent changes in pollinator attraction and fit likely contribute to both the evolutionary divergence within lineages and the convergence among lineages that characterizes pollination guilds.

Tables

Table 2.1 Comparisons of marginal mean (± SE) plant and flower traits among *H. coccinea* populations that differ in flower colour. Values in parentheses indicate the numbers of sites and sampled flowers, inflorescences or plants. *T* values represent the results of score tests. Different letters indicate post-hoc differences between groups (α = 0.05).

	Population type				
Trait	Red	Dark pink	Light pink	Test statistic	*P*
Tepal length (mm)	27.8 ± 0.7 (18, 287)	29.7 ± 1.1 (13, 137)	29.1 ± 0.6 (10, 179)	T_2= 2.92	0.231
Tepal width (mm)	10.5 ± 0.5 ^{A} (18, 287)	11.0 ± 0.7 ^{A} (13, 140)	8.5 ± 0.2 ^{B} (10, 179)	T_2 = 22.28	<0.001
Stamen length (mm)	16.9 ± 0.4 ^{A} (17, 243)	19.9 ± 0.8 ^{B} (10, 118)	19.7 ± 0.7 ^{B} (10, 171)	T_2 = 17.91	<0.001
Style length (mm)	19.6 ± 0.6 ^{A} (17, 244)	24.7 ± 1.2 ^{B} (10, 118)	23.6 ± 0.9 ^{B} (10, 171)	T_2 = 23.23	<0.001
Length of floral tube (mm)	28.9 ± 1.0 ^{A} (18, 287)	32.2 ± 1.3 ^{B} (13, 140)	33.4 ± 0.5 ^{B} (10, 179)	T_2 = 17.20	<0.001

Table 2.1 continued

Trait	Population type			Test statistic	*P*
	Red	Dark pink	Light pink		
Angle of flower face (°)	47.2 ± 3.3 [A] (7, 267)	60.3 ± 0.2 [B] (2, 52)	68.3 ± 1.8 [C] (5, 223)	$T_2 = 34.85$	<0.001
Plant height (cm)	64.3 ± 1.1 (5, 160)	65.2 ± 1.6 (3, 56)	58.2 ± 2.5 (5, 118)	$T_2 = 5.81$	0.055
Flowers per inflorescence	9.8 ± 0.7 (6, 145)	10.5 ± 0.4 (4, 57)	11.2 ± 0.6 (4, 106)	$T_2 = 2.64$	0.268
Open flowers	2.0 ± 0.1 (6, 138)	1.8 ± 0.0 (2, 44)	2.0 ± 0.1 (4, 106)	$T_2 = 6.00$	0.050
Nectar volume (µl)	4.6 ± 0.9 (11, 175)	4.7 ± 0.7 (4, 76)	4.2 ± 0.7 (8, 96)	$T_2 = 0.40$	0.820
Sugar concentration (% m/m)	14.8 ± 0.4 (11,174)	14.6 ± 0.7 (4, 78)	15.3 ± 0.7 (8, 98)	$F_{2,20} = 0.22$	0.801
Nectar sucrose (%)	13.1 ± 3.7[A] (5, 19)	37.4[B] (1, 7)	32.1 ± 11.6[B] (2, 9)	$F_{2,5} = 11.35$	0.014

Table 2.2 Comparisons of marginal mean (± SE) flower traits for seed-derived plants from red- and light pink-flowered populations of *H. coccinea* grown in a common garden. Values in parentheses indicate the numbers of sampled populations and flowers, respectively. *T* values represent the results of score tests.

	Population type			
	Red	Light pink	Test statistic	*P*
Tepal length (mm)	26.9 ± 1.2 (2, 40)	28.2 ± 0.4 (3, 48)	T_1= 0.91	0.339
Tepal width (mm)	12.5 ± 0.1 (2, 40)	9.20 ± 0.3 (3, 48)	T_1 = 93.75	<0.001
Length of floral tube (mm)	22.6 ± 1.0 (2, 35)	29.5 ± 0.2 (3, 46)	T_1 = 42.94	<0.001
Angle of flower face (°)	41.8 ± 5.0 (2, 81)	58.9 ± 4.4 (3, 70)	T_1 = 6.70	0.01
Nectar sucrose (%)	12.2 ± 3.2 (3, 12)	30.6 ± 7.1 (3, 10)	$F_{1,4}$ = 4.91	>0.05

Figures

Figure 2.1

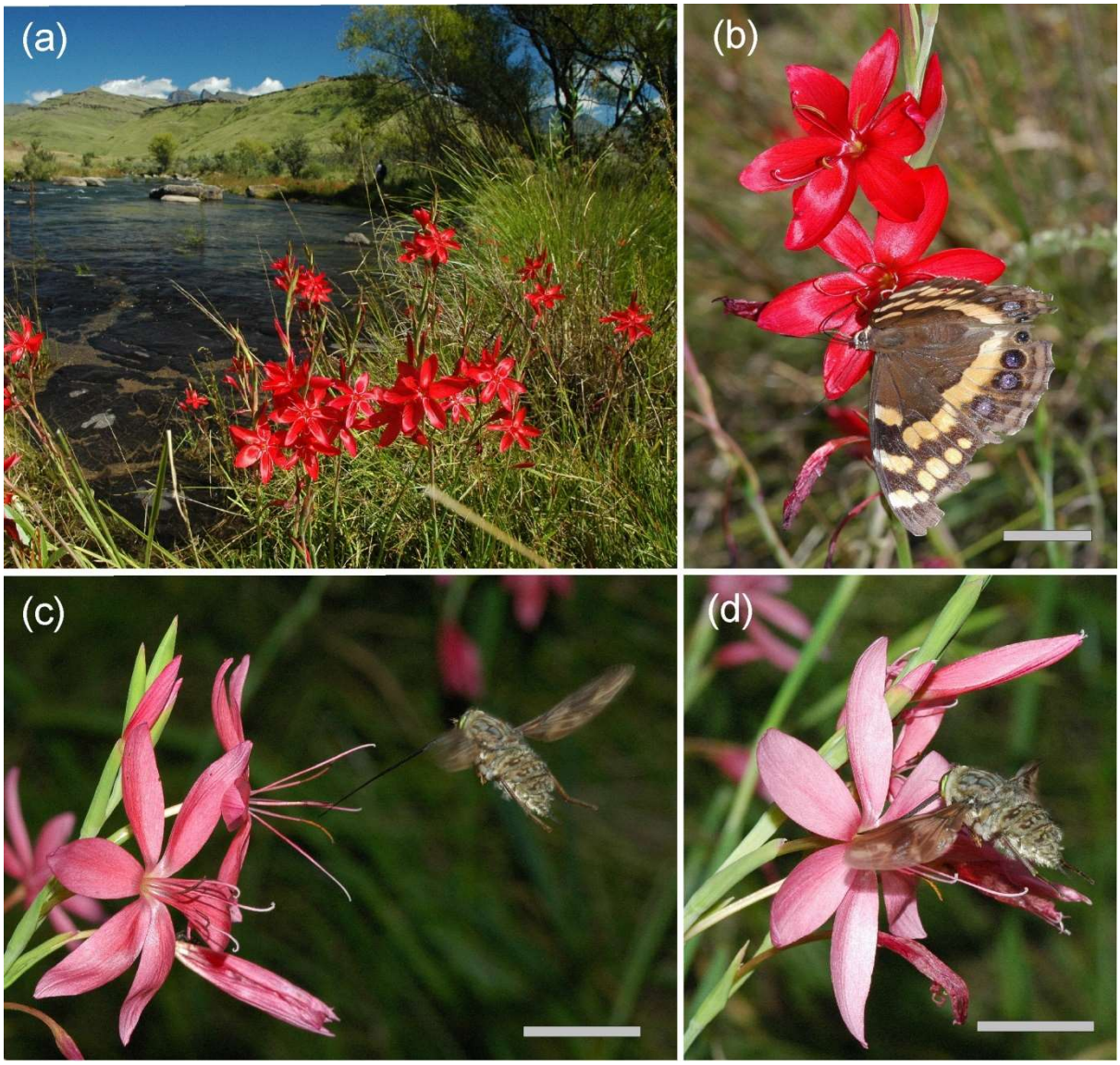

Fig. 2.1 Flower colour, habitat and pollinators of *Hesperantha coccinea*. (a) Flowering plants on the bank of Bushman's River, Bushman's Nek. (b) *Aeropetes tulbaghia* settles to feed on red flowers, Bushman's Nek. (c, d) *Prosoeca ganglbaueri* hovers while visiting pink flowers, Golden Gate. Scale bars 20 mm.

Figure 2.2

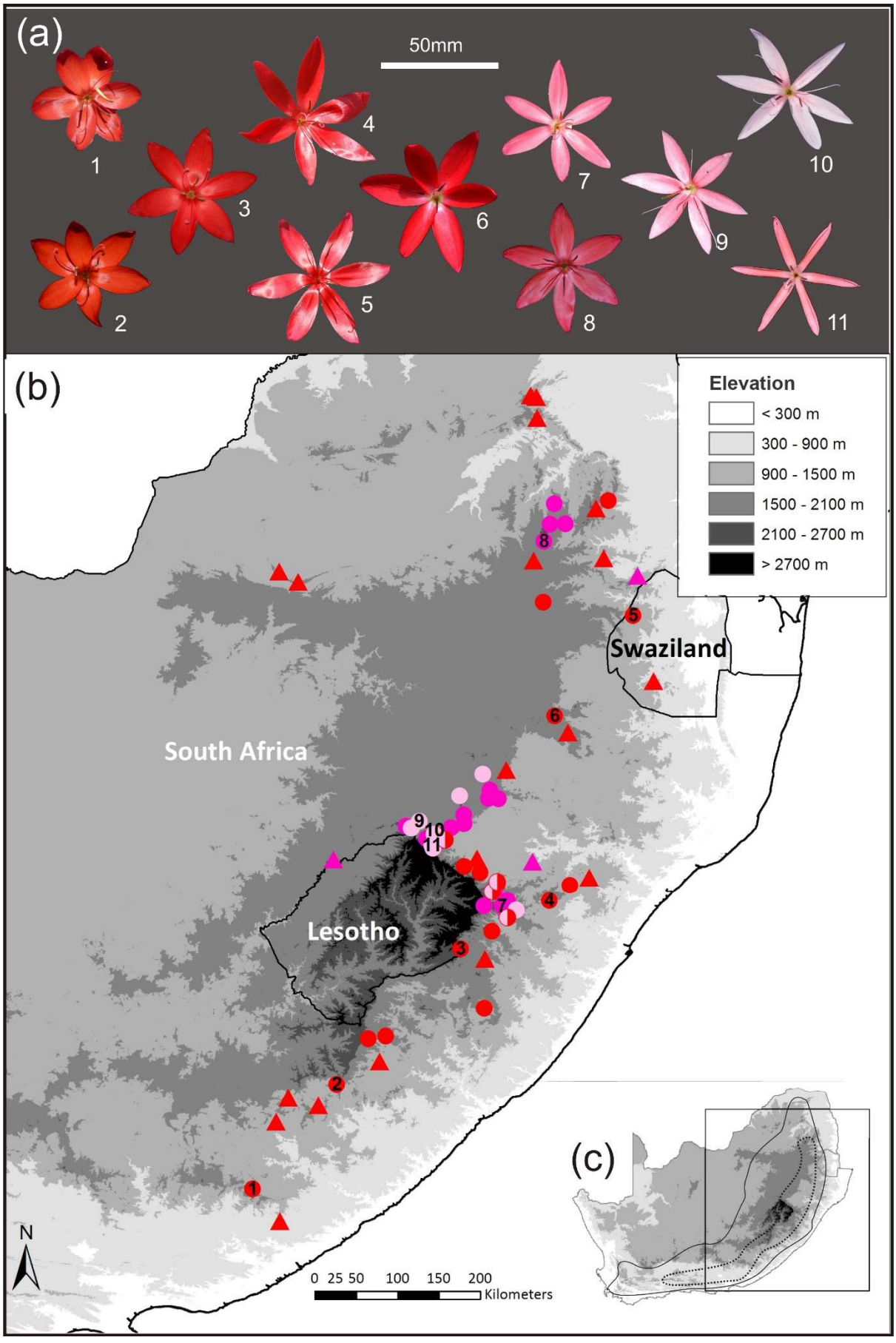
(a)
50mm
1
2
3
4
5
6
7
8
9
10
11
(b)
Elevation
< 300 m
300 - 900 m
900 - 1500 m
1500 - 2100 m
2100 - 2700 m
> 2700 m
Swaziland
South Africa
Lesotho
N
0 25 50 100 150 200
Kilometers
(c)

Fig 2.2 Geographic variation in flower colour and morphology in *H. coccinea*. (a) Flowers from 1, Hogsback; 2, Elliot; 3, Bushman's Nek; 4, Karkloof; 5, Swaziland; 6, Wakkerstroom; (red); 7, Kamberg; 8, Dullstroom; (dark pink); 9, Golden Gate; 10, Devil's Hoek; 11, Mahai (light pink). (b) Flower colour and localities from herbarium records (triangles) and study populations (circles) (See a for flowers from numbered sites). (c) Ranges of *Aeropetes tulbaghia* (solid line) and *Prosoeca ganglbaueri* (dashed line) in South Africa, based on collection localities of specimens from the South African Butterfly Conservation Assessment (Mecenero et al. 2013) and Goldblatt and Manning (2000). Rectangle identifies the region enlarged in (b).

Figure 2.3

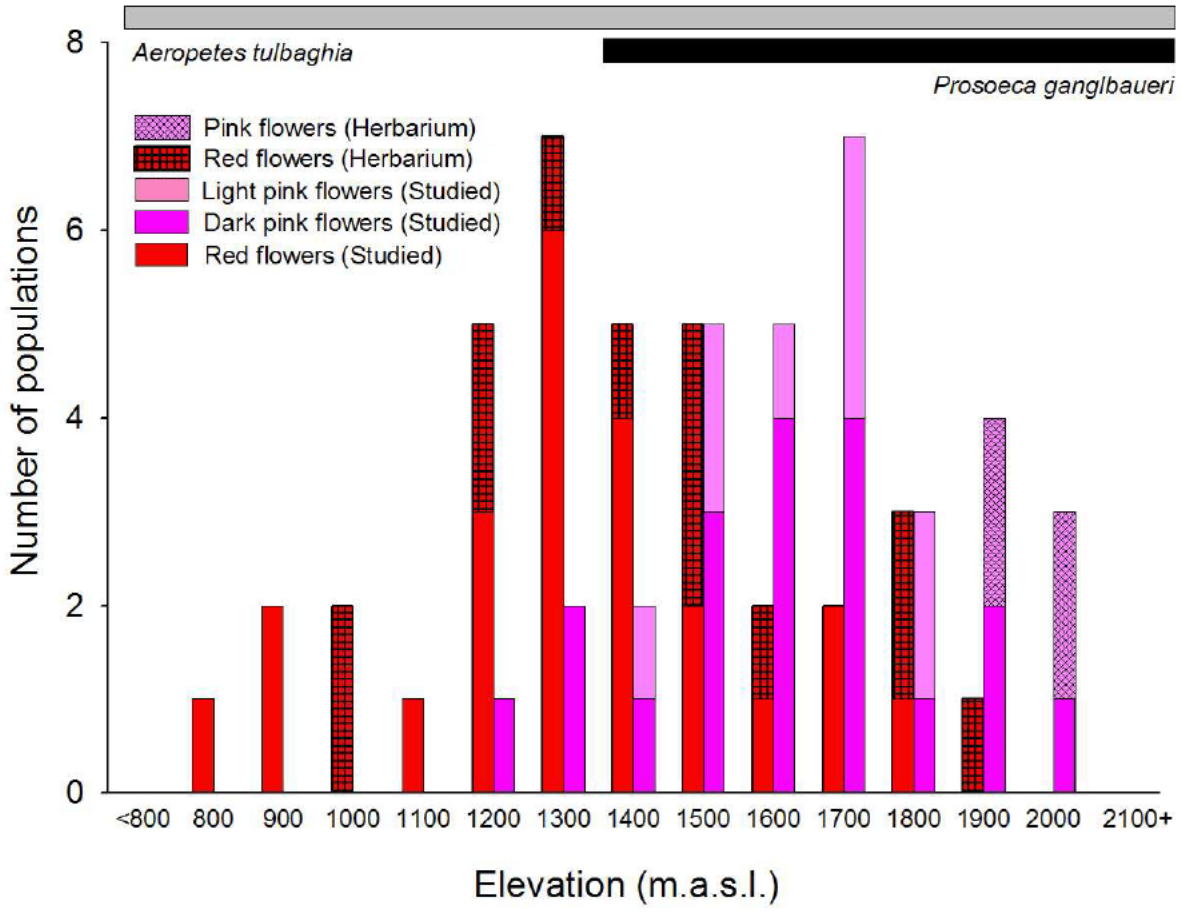

Fig 2.3 Frequency distributions of *H. coccinea* populations with light pink-, dark pink- and red-flowered plants of in relation to elevation. Grey and black bars above indicate the elevation ranges of the primary pollinator species, *Aeropetes tulbaghia* and *Prosoeca ganglbaueri*, respectively.

Figure 2.4

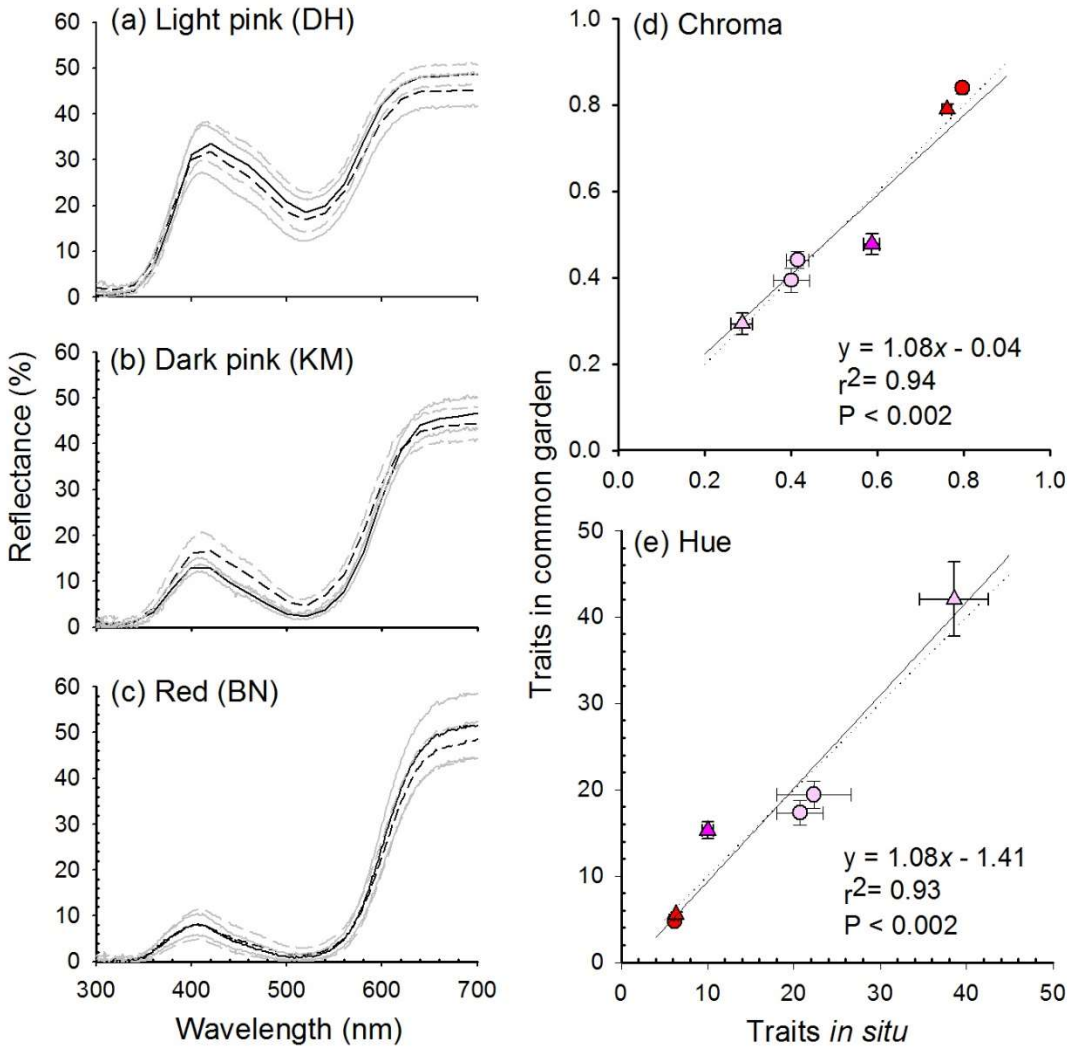

Fig. 2.4 Correspondence of flower colour of *H. coccinea* plants *in situ* and seed-derived plants grown under uniform conditions. Left panels illustrate the mean (black lines) and 95% CI (grey lines) spectral reflectance in the field (solid lines) and in the garden (dashed lines) for plants from (a) Devil's Hoek, light pink flowers; (b) Kamberg, dark pink flowers and (c) Bushman's Nek, red flowers. Right panels present reduced major axis regressions (solid lines) of population means (± SE) for (d) chroma and (e) hue (as defined by Endler, 1990) of flowers cultivated in the common garden and *in situ*. Dotted lines show the 1:1 relation. Light pink, dark pink and red triangles indicate populations shown in panels (a) to (c). Circles indicate additional populations.

Figure 2.5

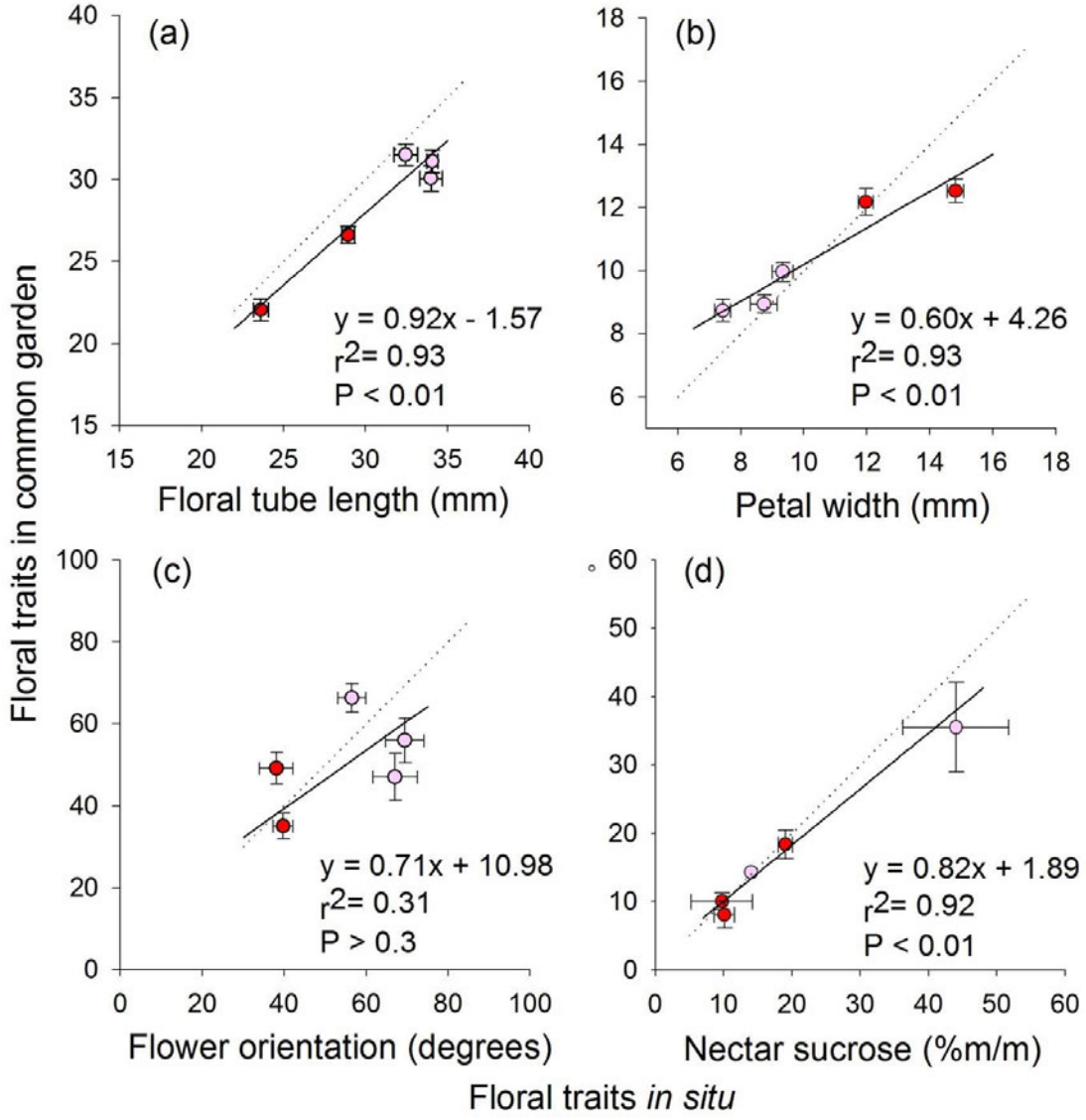

Fig. 2.5 Correspondence of mean (± SE) flower traits of *H. coccinea* plants from five populations *in situ* and for seed-derived plants in a common garden, including (a) floral tube length, (b) tepal width, (c) flower orientation (degrees from horizontal) and (d) nectar sucrose. Solid lines represent the results of reduced major axis regression; dotted lines show the 1:1 relation.

Figure 2.6

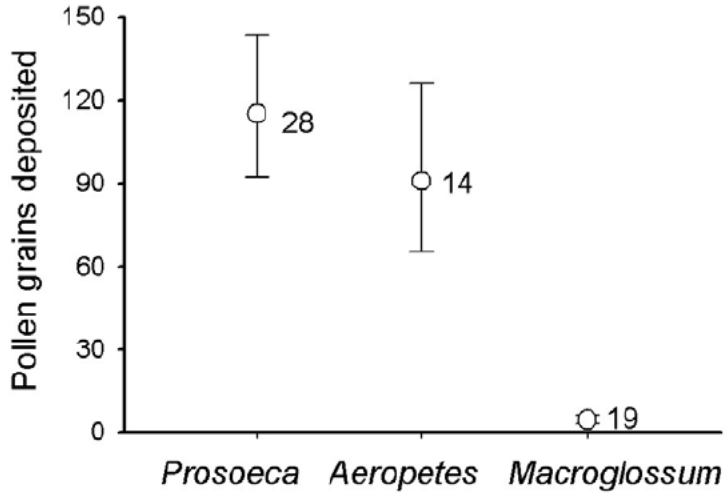

Fig. 2.6 Mean (± SE) pollen deposition on stigmas of virgin flowers following single visits by *Prosoeca ganglbaueri, Aeropetes tulbaghia* and *Macroglossum trochilus*. Numbers of sampled flowers are indicated.

Figure 2.7

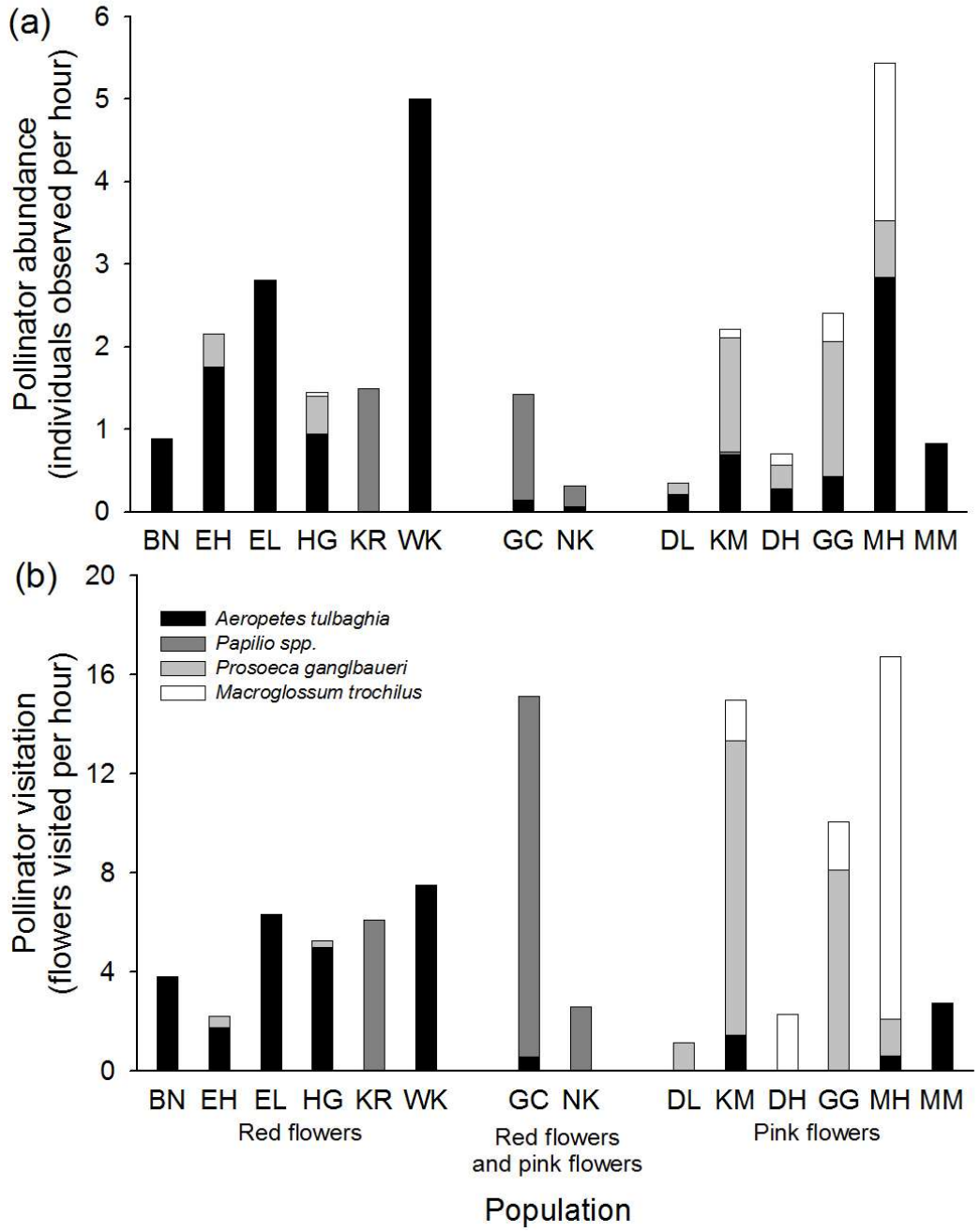

Fig. 2.7 Variation among 14 *Hesperantha coccinea* populations in (a) the numbers of individual butterflies, long-proboscid flies and day-flying hawkmoths observed per hour and (b) the number of flowers visited per hour for each visitor category. See Table A2.1 for site information.

Figure 2.8

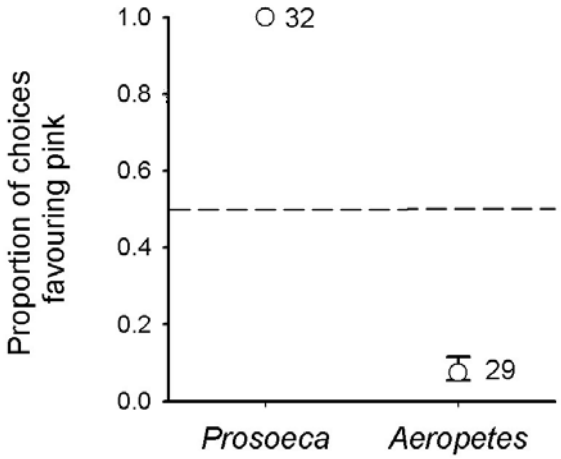

Fig. 2.8 Colour preferences of *A. tulbaghia* and *P. ganglbaueri*, as expressed by the mean (± 95% CI) proportion of choices for pink when offered a pair of model inflorescences with red and pink artificial flowers. Confidence intervals are asymmetrical after back-transformation from the logit scale used for analysis. Numbers of trials are indicated.

Figure 2.9

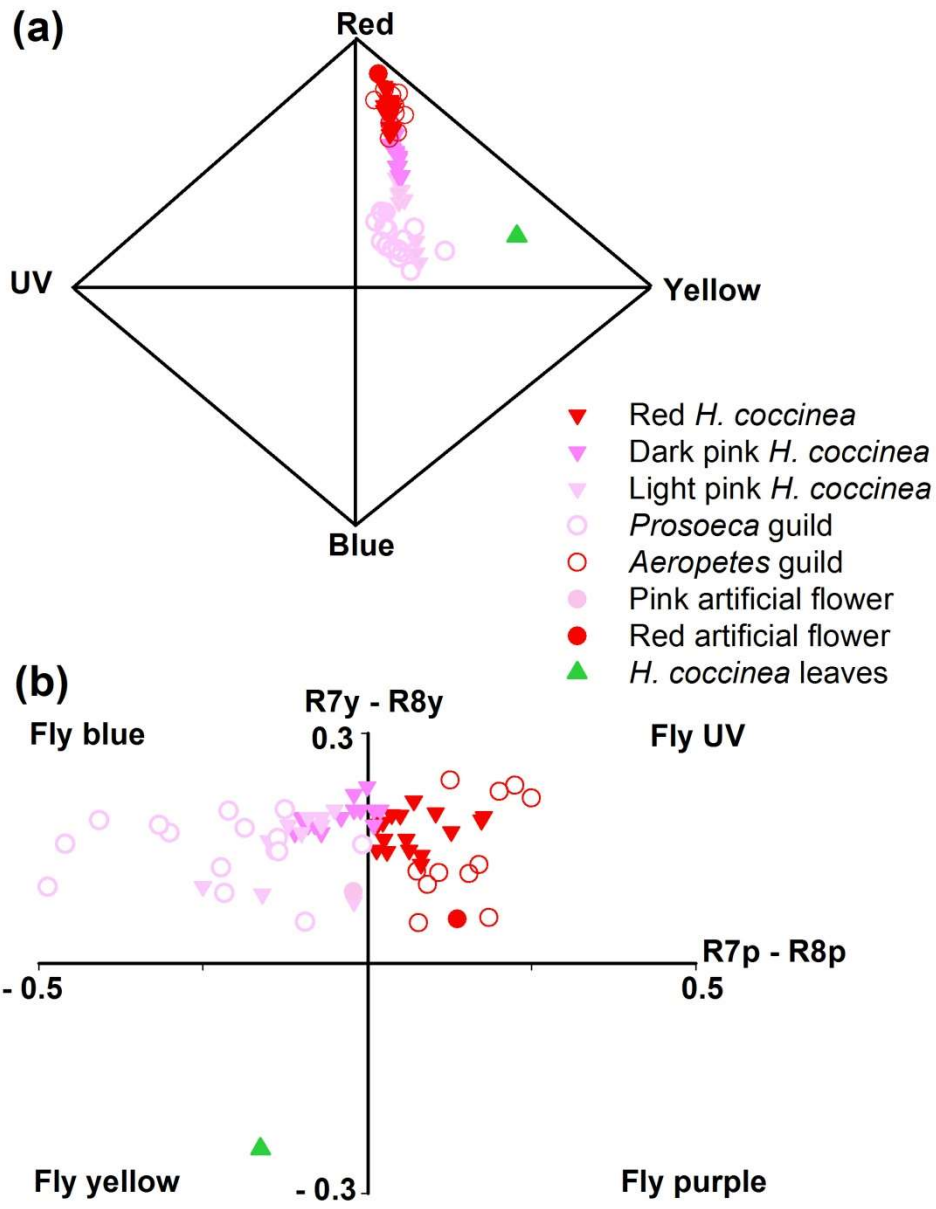

Fig. 2.9 Floral colour loci of *Hesperantha coccinea* populations and species in the pollination guilds of *Aeropetes tulbaghia* and *Prosoeca ganglbaueri*, as perceived according to (a) Endler's (1990) continuous segment classification, in which distance between points represents difference in spectra, and (b) Troje's (1993) categorical model of colour space for fly vision, in which loci within a quadrant are not distinguished. Details and graphical co-ordinates of each population and species are provided in Appendix Tables A2.1 and A2.2.

Appendix

Table A2.1: Site names, abbreviation codes used in figures and tables, flower colour categorization, number of flowers sampled for colour spectra, locality details and colour space co-ordinates for 50 *Hesperantha coccinea* study sites.

Site name	Site code	Colour	*n* flowers	Latitude	Longitude	Elevation	Endler's model x, y		Fly vision model x, y	
Alpine Heath	AP	Red	11	-28.611	29.001	1332	0.10	0.75	0.09	0.15
Bushman's Nek	BN	Red	20	-29.843	29.209	1765	0.09	0.70	0.05	0.20
Carolina	CR	Red	14	-26.075	30.106	1650	0.10	0.68	0.06	0.16
Cathkin	CK	Red	5	-29.008	29.418	1280	0.10	0.70	0.18	0.19
Cathedral Peak	CT	Red	8	-28.943	29.245	1343	0.09	0.80	0.20	0.14
Elands Heights	EH	Red	15	-30.818	28.207	1759	0.10	0.74	0.06	0.15
Elliot	EL	Red	20	-31.313	27.867	1490	0.08	0.81	0.09	0.14
Forest Falls	FF	Red	5	-24.973	30.812	1269	0.10	0.61	0.13	0.18

Table A2.1 continued

Site name	Site code	Colour	*n* flowers	Latitude	Longitude	Elevation	Endler's model x, y		Fly vision model x, y	
Hogsback	HG	Red	18	-32.445	26.950	1371	0.10	0.64	0.11	0.09
Inverness	IN	Red	11	-29.149	30.394	1345	0.09	0.73	0.04	0.17
Karkloof	KR	Red	20	-29.318	30.171	1175	0.08	0.73	0.17	0.19
Kokstad	KK	Red	11	-30.486	29.465	1417	0.09	0.75	0.05	0.15
Pitseng	PI	Red	10	-30.789	28.395	1443	0.08	0.71	0.07	0.21
Sani Pass	SN	Red	4	-29.653	29.546	1434	0.10	0.69	0.20	0.16
Swaziland	SZ	Red	7	-26.218	31.078	1401	0.10	0.64	0.05	0.19
Wakkerstroom	WK	Red	21	-27.309	30.231	1808	0.11	0.63	0.04	0.19
Wembezi Lucky	WL	Red	10	-29.137	29.606	1533	0.11	0.72	0.10	0.10

Table A2.1 continued

Site name	Site code	Colour	*n* flowers	Latitude	Longitude	Elevation	Endler's model x, y		Fly vision model x, y	
Collingspas	CO	Dark pink	6	-28.208	29.620	1739	0.12	0.48	-0.10	0.19
Dorpspruit	DS	Dark pink	5	-25.223	30.344	1540	0.11	0.62	0.02	0.20
Dullstroom	DL	Dark pink	14	-25.414	30.112	2013	0.11	0.57	-0.02	0.20
Fiko Patso	FP	Dark pink	4	-28.644	28.838	1749	0.13	0.44	-0.11	0.17
Giants Location	GL	Dark pink	5	-29.185	29.580	1432	0.12	0.53	-0.05	0.20
Golden Huts	GH	Dark pink	15	-28.506	28.616	1916	0.13	0.51	-0.08	0.18
Happy Home	HH	Dark pink	7	-28.115	29.527	1872	0.11	0.57	-0.09	0.18
Highmoor	HM	Dark pink	5	-29.329	29.675	1586	0.11	0.61	-0.01	0.20
Kamberg	KM	Dark pink	20	-29.38	29.659	1703	0.10	0.57	0.00	0.23
Kerkenberg	KB	Dark pink	6	-28.522	29.105	1670	0.13	0.49	0.01	0.18

Table A2.1 continued

Site name	Site code	Colour	*n* flowers	Latitude	Longitude	Elevation	Endler's model x, y		Fly vision model x, y	
Kleinmooi	KO	Dark pink	63	-29.319	29.716	1534	0.10	0.65	0.02	0.20
Loteni	LT	Dark pink	7	-29.378	29.460	1548	0.12	0.61	0.01	0.20
Maartenshoop	MT	Dark pink	8	-25.007	30.217	1329	0.10	0.65	-0.02	0.20
Netherby	NB	Dark pink	3	-28.476	29.247	1652	0.13	0.48	-0.07	0.18
Potspruit	PS	Dark pink	6	-25.229	30.181	1937	0.11	0.59	-0.02	0.22
Renny Lynn	RL	Dark pink	4	-29.391	29.738	1636	0.11	0.55	-0.09	0.18
Rensbergkop	RK	Dark pink	2	-28.384	29.243	1675	0.12	0.54	-0.09	0.18
Rugged Glen	RG	Dark pink	4	-28.666	28.992	1364	0.14	0.44	-0.07	0.17
Skeurklip	SK	Dark pink	6	-28.203	29.510	1711	0.13	0.52	-0.04	0.19
Bezuidenhout	BZ	Light pink	9	-28.177	29.196	1680	0.15	0.34	-0.15	0.16

Table A2.1 continued

Site name	Site code	Colour	*n* flowers	Latitude	Longitude	Elevation	Endler's model x, y		Fly vision model x, y	
Cavern Berg	CB	Light pink	3	-28.634	28.961	1490	0.20	0.08	-0.02	0.08
Candle	CQ	Light pink	4	-28.527	28.672	1791	0.13	0.36	0.07	0.19
Devil's Hoek	DH	Light pink	20	-28.714	28.934	1540	0.19	0.17	-0.25	0.10
Golden Gate	GG	Light pink	20	-28.508	28.620	1931	0.14	0.38	-0.10	0.17
Mahai	MH	Light pink	25	-28.696	28.906	2047	0.13	0.33	-0.12	0.18
Metsimatso	MM	Light pink	14	-28.592	28.919	1826	0.19	0.12	-0.16	0.09
Normandien	ND	Light pink	4	-27.941	29.447	1732	0.13	0.36	-0.07	0.18
Nottingham Road	NR	Light pink	4	-29.421	29.811	1681	0.13	0.37	-0.09	0.19
Puthaditjaba	PJ	Light pink	20	-28.470	28.760	1712	0.12	0.43	-0.05	0.20

Table A2.1 continued

Site name	Site code	Colour	*n* flowers	Latitude	Longitude	Elevation	Endler's model x, y		Fly vision model x, y	
Giants Castle	GC	Light pink to red	22	-29.220	29.554	1535	n/a	n/a	n/a	n/a
Nkolweni	NK	Light pink to red	32	-29.507	29.717	1569	n/a	n/a	n/a	n/a
Tugela	TG	Light pink to red	12	-28.655	29.041	1234	n/a	n/a	n/a	n/a
White Mountain	TH	Light pink to red	5	-29.112	29.606	1533	n/a	n/a	n/a	n/a

Table A2.2: Species in the pollination guilds of *Aeropetes tulbaghia* (a1-11) and *Prosoeca ganglbaueri* (b1-16) and their colour characteristics based on Endler's (1990) continuous segment classification and Troje's (1993) categorical (fly) model. Labels in the spectrum column correspond to spectra shown in Figure A2.1a and b. Guild membership is according to Johnson and Bond (1994) (*A. tulbaghia*) and Goldblatt and Manning (2000) and Anderson and Johnson (2009) (*P. ganglbaueri*). Species for which additional spectra from Johnson and Bond (1994) were used are marked with an asterisk.

Spectrum	Species, *n* sites, *n* flowers	Endler x	Endler y	Fly p	Fly y
a1	*Tritoniopsis triticaea** 2, 8	0.12	0.73	0.09	0.10
a2	*Disa uniflora** 2, 6	0.09	0.74	0.08	0.12
a3	*Kniphofia uvaria* 2, 10	0.22	0.54	0.08	0.05
a4	*Disa ferruginea** 1, 6	0.10	0.66	0.11	0.12
a5	*Brunsvigia marginata** 1, 5	0.10	0.59	0.13	0.24
a6	*Gladiolus nerinoides**	0.08	0.80	0.16	0.12
a7	*Gladiolus cardinalis** 1, 9	0.11	0.77	0.17	0.13
a8	*Nerine sarniensis**	0.13	0.78	0.19	0.06
a9	*Cyrtanthus elatus** 1, 6	0.12	0.70	0.22	0.23
a10	*Gladiolus saundersii* 1, 3	0.13	0.62	0.20	0.22
a11	*Crassula coccinea** 1, 11	0.05	0.75	0.25	0.22
b1	*Brownlea macroceras* 2, 16	0.17	0.05	-0.22	0.09
b2	*Brunsvigia grandiflora* 2, 18	0.14	0.12	-0.14	0.15
b3	*Cycnium racemosum* 3, 21	0.13	0.13	-0.22	0.13
b4	*Dianthus basuticus* 1, 7	0.09	0.22	-0.46	0.16
b5	*Disa amoena* 2, 15	0.08	0.23	-0.13	0.20
b6	*Disa erecta* 1, 4	0.09	0.15	-0.19	0.18
b7	*Disa nivea* 1, 16	0.05	0.25	-0.09	0.05
b8	*Disa rhodantha* 1, 12	0.07	0.29	-0.19	0.18
b9	*Gladiolus microcarpus* 1, 17	0.13	0.10	-0.14	0.15
b10	*Gladiolus oppositiflorus* 2, 10	0.18	0.22	-0.01	0.15
b11	*Hesperantha grandiflora* 1, 10	0.11	0.14	-0.30	0.17

Table A2.2 continued

Spectrum	Species, *n* sites, *n* flowers	Endler x	Endler y	Fly p	Fly y
b12	*Hesperantha scopulosa* 2, 14	0.07	0.17	-0.32	0.18
b13	*Hesperantha woodii* 1, 7	0.09	0.15	-0.21	0.20
b14	*Nerine bowdenii* 1, 5	0.15	0.18	-0.49	0.10
b15	*Watsonia wilmsii* 1, 13	0.07	0.28	-0.41	0.19
b16	*Zaluzianskya microsiphon* 3, 18	0.29	0.13	-0.14	0.16

Figure A2.1

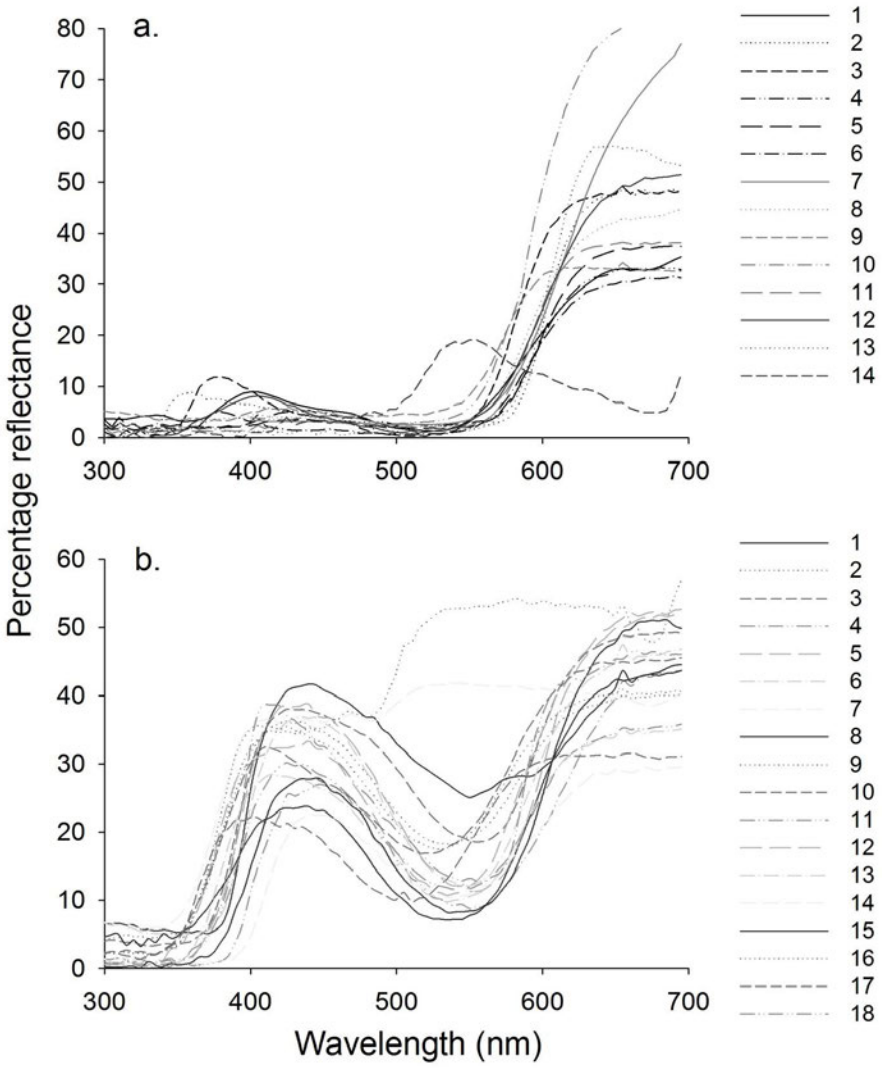

Fig. A2.1 Spectral reflectance of species in pollination guilds, corresponding artificial flowers, and leaves. (a) *Aeropetes tulbaghia* guild: 1. *Brunsvigia marginata;* 2. *Crassula coccinea;* 3. *Cyrtanthus elatus;* 4. *Disa ferruginea;* 5. *D. uniflora;* 6. *Gladiolus cardinalis; 7. G. neriniodes;* 8. *G. saundersii; 9. Kniphofia caulescens;* 10. *Nerine sarniensis;* 11. *Tritoniopsis triticaea;* 12. Representative red population (BN); 13. Red artificial flower; 14. *H. coccinea* leaves. (b) *Prosoeca ganglbaueri* guild: 1. *Brownleea macroceras;* 2. *Brunsvigia grandiflora;* 3. *Cycnium racemosum;* 4. *Dianthus zeyheri*; 5. *Disa amoena;* 6. *D. erecta;* 7. *D. nivea;* 8. *D. rhodantha;* 9. *Gladiolus microcarpus,* 10. *G. oppositiflorus.* 11. *Hesperantha grandiflora;* 12. *H. scopulosa;* 13. *H. woodii;* 14. *Nerine bowdenii;* 15. *Watsonia wilmsii;* 16. *Zaluzianskya microsiphon,* 17. Representative light pink population (DH); 18. Pink artificial flower. See Table A2.2 for the summary colour characteristics for each plant species based on Endler's (1990) continuous segment classification and Troje's (1993) categorical (fly) model.

CHAPTER 3: LOCAL ADAPTATION TO DIFFERING POLLINATORS UNDERLIES DIVERGENCE IN FLORAL COLOUR AND MORPHOLOGY IN *HESPERANTHA COCCINEA* (IRIDACEAE)

1

Abstract

Geographical co-variation of floral traits and pollinators is frequently ascribed to local adaptation of plants to different pollinators, but this hypothesis is seldom tested experimentally. In *Hesperantha coccinea* (Iridaceae), a species which combines specialised pollination by insects and facultative autogamy, plants in pink-flowered populations of the northern Drakensberg region tend to be visited by long-proboscid flies, whereas those in morphologically-distinct red-flowered populations in the Southern Drakensberg tend to be visited more often by butterflies. I translocated plants of both morphs to sites in both environments (northern and southern Drakensberg) to assess the context dependence of pollinator attraction and the pollinator-mediated components of pollen deposition and seed production. Choice tests revealed clear preferences for red morphs by butterflies and for pink morphs by long-proboscid flies. Single-visit pollen deposition was highest when long-proboscid flies visited pink morphs and when butterflies visited red morphs. In both environments, flowers that matched the colour of local morphs were visited more frequently and received more pollen during their lifespans than did flowers of foreign morphs. Finally, in experiments involving reciprocal translocation of emasculated plants between the two environments, local plants produced more seeds than plants of introduced morphs, indicating local adaptation. This study provides strong evidence that floral divergence among populations reflects local adaptation to different pollinators, and uniquely identifies both colour signals and mechanical fit as important components of local floral adaptation.

Key words: *Aeropetes tulbaghia, Prosoeca ganglbaueri*, pollination ecotypes, floral evolution, pollinator-effectiveness, pollinator-attraction.

Introduction

Adaptation to different pollinators generates much of the spectacular diversity of floral forms among the angiosperms (Darwin 1877, Crepet 1984, Dodd et al. 1999, Vamosi and Vamosi 2010). Among species, floral traits correlate with pollinator groups (Armbruster 1985, Johnson et al. 1998, Whittall and Hodges 2007, Smith and Rausher 2008, Martén-Rodrìguez et al. 2010) and as much as a quarter of angiosperm speciation events may have involved shifts in pollination system (van der Niet and Johnson 2012). Grant and Grant (1965) and Stebbins (1970) proposed that pollinator-driven divergence is initiated by geographic variation in pollinator abundance, which promotes local floral adaptation to the most frequent and effective local pollinators ("Grant-Stebbins model", Johnson 2006). Given that animal pollinators often differ in sensory preferences, behaviour and morphological characteristics, this adaptation is expected to involve correlated suites of floral traits, including those involved in pollinator attraction (signals and rewards) and pollen exchange with attracted pollinators ("pollinator fit") (Stebbins 1970, Fenster et al. 2004, Rosas-Guerrero et al. 2014).

Pollinator-mediated divergence consistent with the Grant-Stebbins model is suggested by "pollination ecotypes", or associated differences among populations in pollinators and reproductive traits (e.g. Robertson and Wyatt 1985, Arroyo and Dafni 1995, Johnson 1997, reviewed in van der Niet et al. 2014a). However, most studies of pollination ecotypes have simply identified trait-environment correlations. Therefore, they do not fully demonstrate that variation is adaptive and pollinator-mediated, or characterize the mechanisms or environmental basis of divergence (Heslop-Harrison 1958, cf. Gould and Lewontin 1979, Herrera et al. 2006, Leimu and Fischer 2008, Nuismer et al. 2010). Floral divergence frequently reflects adaptation that is not pollinator-driven (reviewed in Strauss and Whittall 2006) and pollinator foraging preferences can result in associations between floral traits and pollinators in the absence of underlying differences in pollinator abundance ("ecological fitting" sensu Janzen 1985, Herrera et al. 2006). Indeed, several ecotype studies of correlations between traits and pollination environments are based on observations of pollinators and did not distinguish ecological fitting from qualitative or quantitative turnover in pollinator abundance (e.g. Armbruster 1985, Robertson and Wyatt 1985, cf. Valiente-Banuet et al. 2004, Boberg et al. 2014, Sun et al. 2014, van der Niet et al. 2014b). Thus, the environmental basis of divergence in these studies is unclear.

The differences in pollinator characteristics that mediate divergence are also typically inferred from correlations between floral traits and pollinators. For example, divergence

mediated by pollinator fit is often inferred from co-variation of flower and pollinator dimensions (e.g. Robertson and Wyatt 1985, Johnson 1997, Boberg et al. 2014, van der Niet et al. 2014b) and the importance of pollinator attraction is inferred from co-variation of advertising traits and pollinators among population (e.g. Peter and Johnson 2014, Sun et al. 2014, van der Niet et al. 2014b). However, effects of pollinator morphology or preference on morph fitness are rarely verified in pollination-ecotype studies, so alternative causes of correlations are not excluded (but see Johnson and Steiner 1997, Newman et al. 2012, Boberg et al. 2014). Therefore, despite its intuitive appeal and the accumulated correlational evidence, the Grant-Stebbins model remains to be tested directly. Such a test requires evidence that intraspecific floral divergence is adaptive, mediated by pollinators and driven by differences in pollinator abundance.

The adaptive significance of trait divergence has frequently been tested by comparing the performance of alternative morphs in contrasting environments using reciprocal translocation experiments (reviewed in Schluter 2000, Leimu and Fischer 2008, Hereford 2009). A performance advantage of local plants relative to foreign plants provides evidence that trait divergence reflects adaptation to local environmental conditions (Kawecki and Ebert 2004, Hargreaves et al. 2014). Replication of reciprocal translocation experiments between several pairs of sites characterised by the same environmental differences have been used to identify likely drivers of local adaptation (tests of "parallel local adaptation", sensu Kawecki and Ebert 2004, e.g. Berglund et al. 2004, Briscoe Runquist and Moeller 2014, also see Hargreaves et al. 2014). Reciprocal translocation can identify local adaptation in particular sites or habitats, but not which aspects of these habitats drive divergence, which instead requires experimental manipulation of hypothesized environmental drivers (such as soils or pollinators) and measurement of the effects on morph performance (e.g. Berglund et al. 2004). Most reciprocal translocation experiments that contrasted pollination environments provided only partial evidence for the role of pollinators in divergence (Streisfeld and Kohn 2007, Boberg et al. 2014, Sun et al. 2014). In only two such studies did the local morph outperform the foreign morph in both environments (Gómez et al. 2009, Newman et al. 2012), thus meeting the strictest criterion of adaptive divergence (Kawecki and Ebert 2004, Hereford 2009). Of these studies, only Newman et al. (2012) verified the mechanism and environmental basis of pollinator-mediated fitness differences. However, Gómez et al. (2009) and Newman et al. (2012) considered only the rates of pollinator visitation, which likely does no accurately represent reproductive success. Thus, insight into the processes underlying

floral divergence among populations awaits coordinated analysis of both local adaptation and the mechanisms of pollinator-mediated selection.

My studies of the southern African irid *Hesperantha coccinea* (Backh. and Harv.) Goldblatt and J.C. Manning demonstrated that population differences in flower colour, morphology and nectar traits are genetically based and associated with differences in pollinator assemblages (Chapter 2). In pink-flowered populations, a long-proboscid fly, *Prosoeca ganglbaueri* (Lichtwardt), is the most common visitor, whereas in red-flowered populations the butterfly *Aeropetes tulbaghia* (Linneaus) visits most frequently (Johnson and Bond 1994, Goldblatt et al. 2004, Chapter 1). Floral traits that differ between floral morphs of *H. coccinea* could affect pollinator attraction by acting as signals (flower colour and petal width, Jersakova et al. 2012, Newman et al. 2012) and also influencing the fit between floral reproductive structures and pollinators (floral tube length, Nilsson 1988, Bloch and Erhardt 2008, Anderson and Johnson 2009, and orientation, Ushimaru et al. 2009). Indeed, *P. ganglbaueri* and *A. tulbaghia* have strongly contrasting colour preferences for pink and red, respectively (Chapter 2), and also differ morphologically and behaviourally, such that differences in their relative abundance among sites could drive colour and morphological divergence in *H. coccinea*. Thus, *H. coccinea* is an excellent case to explore the role of pollinator-adaptation in floral divergence.

I hypothesized that floral divergence in *H. coccinea* resulted from local adaptation to differential visitation by flies and butterflies among sites, and tested four associated predictions. First, if selection acts on colour signals, fly and butterfly pollinators should exhibit contrasting preferences for floral morphs in mixed arrays. Second, if selection acts via morphological fit, then single visits by pollinators associated with a particular floral morph should deposit more pollen than those by pollinators associated with the contrasting floral morph. Third, if pollination mediates selection, local morphs should receive more pollinator visits than introduced morphs and correspondingly receive more pollen on stigmas. Finally, owing to local adaptation, plants of the local floral morph should produce more seeds than introduced plants of the alternative morph following reciprocal translocation.

Materials and Methods

Study species

Hesperantha coccinea is a perennial geophyte that grows along montane watercourses from the Drakensberg escarpment in the Eastern Cape of South Africa to the highlands of eastern Zimbabwe (Goldblatt and Manning 1996b). Flowers of *H. coccinea* range from pink to red (Goldblatt and Manning 1996b, Goldblatt 2003, Chapter 1). Although both colours involve similar reflectance in the red region (580 and 620 nm), red flowers have average reflectance <10% of total reflectance in the blue region (400 and 500 nm), whereas for pink flowers this region contributes up to 50% of total reflectance (Chapter 2). Among *H. coccinea* populations, differences in flower colour correlate with genetically-based differences in functional floral depth, petal width and flower orientation (Chapter 2). As flower colour is the most obvious feature distinguishing these morphs, I refer to them as pink- and red-flowered for convenience; however, these names also connote the associated morphological differences. Compared to red-flowered populations, plants in pink-flowered populations have flowers with more elongated floral tubes, stamens and styles, narrow petals, a more vertically oriented flower face and higher nectar sucrose content. Red-flowered populations dominate the southern Drakensberg region and are pollinated almost exclusively by butterflies, primarily *Aeropetes tulbaghia*, whereas pink-flowered populations are mostly localised in the northern Drakensberg region and are pollinated by the long-proboscid fly *Prosoeca ganglbaueri* (Chapter 2).

Study sites

Experiments were conducted at two sites (Golden Gate and Mahai, henceforth GG and MH) in the northern Drakensberg mountain region where pink-flowered populations occur (hereafter pink environment) and two sites (Elliot and Elands Heights, henceforth EL and EH) in the southern Drakensberg region where red-flowered populations occur (hereafter red environment: see Table 3.1 for details of study sites). Plants and inflorescences used in translocation experiments were collected from these sites and an additional, later-flowering site in the red environment, Bushman's Nek (BN). Study sites within environments are located within approximately 80 km of each other and are approximately 350 km from sites in the other environment (for locality details see Table 3.1). Experiments were conducted during peak flowering, from the end of February to the beginning of April, 2014.

Pollinator preference and effectiveness

To assess pollinator preference and single-visit pollen deposition, pairs of cut inflorescences from a foreign pink-flowered population (MH) and a red-flowered population (EH) were presented to pollinators in different pink- (GG) and red-flowered (EL) populations (see Table 3.2). Inflorescences were transported between populations with their cut stems in wet florist's foam. Pairs of two emasculated, unpollinated inflorescences, one of each colour morph, were placed in water-filled florist spikes separated by 50 cm at the end of a 1-m stick (Thomson 1988, as modified by Johnson 2000). Each pair was then presented to individual *P. ganglbaueri* and *A. tulbaghia* as they visited flowers at sites GG and EH. To quantify preference of each pollinator species, I recorded the colour of the first flower visited by every individual. To quantify per visit pollen deposition, morph colour and pollinator species were noted after each visit to an unpollinated flower and the stigma was mounted in fuchsin gel on a glass slide and covered with a cover slip. Pollen grains were subsequently counted at 100× magnification. Observations of pollinator preference were conducted in fine weather during March 2014 (GG – 31 h observation during 5 days; EH – 26 h observation during 4 days).

Common environment experiments

I used a common environment (≈ common garden) experiment to compare lifetime pollination success of flowers of the two morphs in natural populations in the red and pink environments. Test inflorescences of each morph were collected from source populations and transported to a common context population. Multiple pairs of inflorescences, with one matching the local floral morph and one of the contrasting morph, were arrayed in context populations in both the red and pink environments (details of populations and sampling in Table 3.2). Each inflorescence pair was placed in a water-filled vase made from a clear plastic 2-L bottle and pairs were positioned approximately 60 cm apart along the river bank. Experimental flowers were exposed to natural pollination for approximately one week during peak flowering. To ensure that pollen receipt was attributable only to pollinator activity (the hypothesized agents of selection) and not late-acting self-pollination at context sites, the buds of all experimental flowers were emasculated, marked with coloured wire, and covered with fine mesh to exclude pollinators until the experiment began. This procedure also eliminated the risk of genetic contamination of context populations. During experimental trials at the GG (pink environment) and EH (red environment) sites, I observed inflorescence visitation during the observation times described above, recording the total number of flowers of each

morph visited by all pollinators. To quantify pollen receipt, senesced experimental flowers were collected and softened in a 70% ethanol solution to enable dissection of stigmas from wilted floral tissue. Softening may dislodge pollen from stigmas, particularly ungerminated grains. However, pollen germination did not differ statistically among morphs (Cozien, unpublished data), so any loss due to softening likely did not affect results. Stigmas were mounted in fuchsin gel on glass slides to stain pollen grains and pollen grains were counted at 100×. I could not assess pollen receipt for the EH site because a flood washed the experimental inflorescences away.

Reciprocal translocations

To assess evidence for local adaptation, I conducted three translocation experiments involving pairs of sites from the red and pink environments (see Table 3.3) and quantified seed set following natural pollination. Translocation was reciprocal for Experiments 1 and 2, which were conducted simultaneously, whereas for Experiment 3 plants from pink environment (MH) were not moved to the red environment (BN) because flowering at BN had passed its peak and too few plants remained flowering to conduct the experiment. To move individuals between sites (but not within sites), plants with intact root balls were placed in 23-cm diameter pots, which were then dug into the river bank among plants of the local morph. Roots of translocated plants were positioned below the water surface, consistent with the natural habit of the species. All flowers and buds on experimental local and foreign plants were emasculated (to ensure that results reflect pollinator-mediated processes and not facultative self-pollination) and labelled with coloured wire, but local experimental plants were otherwise unmanipulated. Plants were exposed to natural pollination for two to three weeks. Once all experimental flowers had wilted and fruit development was clearly evident from swollen ovaries, inflorescences were collected and the number of developed seeds was counted for every experimental flower Flowers that did not develop fruits were assigned zero seeds. Developed seeds were clearly distinguishable from the small (<0.2 mm diameter) colourless undeveloped ovules by turgidity, their green to brown colour and size (>1 mm diameter).

To assess whether translocation affected physiological potential for seed production and to implicate pollination in differences in reproductive success, I also calculated a measure of overall “pollinator limitation” (the difference between actual and potential seed production of emasculated flowers) using pollen supplementation (cf. Bierzychudek 1981). At one site

for Experiments 1 (GG) and 3 (MH) and both sites for Experiment 2 (GG and EH), one flower on multiple plants of the local and foreign morphs was hand-pollinated with pollen collected from several plants in the experimental site (see Table 3.3 for sample sizes). The resulting seeds were counted and these counts were compared with seed production of the naturally-pollinated experimental plants.

To test for post-pollination reproductive barriers that could cause differences in reproductive success between local and foreign morphs in translocation experiments, hand-pollination was used to assess inter-morph compatibility. Plants grown from wild-collected seed from two populations of each morph were maintained in a pollinator-free greenhouse at the Pietermaritzburg campus of the University of KwaZulu-Natal. Emasculated flowers were randomly assigned to three pollination treatments involving pollen from the same population, a different population of the same morph, or a population of the contrasting morph. Developed seeds were quantified as described above. For sampling details see Appendix, Table A1.

Statistical analyses

Statistical analyses involved generalised linear models (GLM) (McCullagh and Nelder 1989) implemented in SPSS 22 (IBM Inc.) and generalised linear mixed models (GLMM) (Stroup 2013) implemented in SAS 9.4 (the glmmix procedure, SAS Institute Inc., 2013). Counts of seeds, pollen grains and flowers visited were analysed with negative binomial distributions and log link functions, whereas analysis of the proportion of choices for the local floral morph considered a binomial distribution and logit link function. Hypothesis tests for fixed effects involved likelihood-ratio tests (G) (Littell et al. 2006) in analyses of independent data (GLM), score tests (T) (Boos 1992) for analyses that accounted for correlated responses with generalised estimating equations (GEE) (Liang and Zeger 1986) and F-tests for generalised linear mixed models (Stroup 2013). For all analyses, significant interactions between floral morph and environment or pollinator were explored further using multiple comparisons, with $\alpha = 0.05$ for individual planned contrasts between local and foreign floral morphs within environments or pollinator species, and the Dunn-Šidák procedure for all other comparisons (Kirk 1995).

Analyses of pollinator preference and per-visit pollen deposition involved inflorescences from the choice trials. Differences in the proportion of first visits to pink-flowered plants in choice tests were analysed with GEE and an exchangeable correlation

matrix to account for repeated observations of individual pollinators (Liang and Zeger 1986). Pollinator species and context environment were crossed, fixed factors. Each pollinator species' preference for or against flowers of the local floral morph was assessed by testing whether the mean logit of first visits to pink inflorescences differed significantly from 0 (corresponding to an equal-choice proportion of 0.5). Counts of the number of pollen grains deposited per visit were analysed using GLM, with morph and pollinator as crossed, fixed factors and site nested within morph as a fixed factor.

I analysed pollinator visitation and pollen deposition for the translocation experiment with GLMs, with morph and site included as crossed, fixed factors. For pollen receipt per inflorescence the analysis also considered site nested within environment, morph and environment, their interaction and site nested within environment as fixed factors. The analyses of visits per hour, pollen receipt per inflorescence, and seed production per plant respectively included the ln-transformed numbers of experimental flowers per array, inflorescence and plant as offset variables to obtain standardized measures and to account for variation in sampling intensity.

Seed production per flower during the reciprocal transplant experiments was analysed with two sets of two analyses to accommodate the incomplete sampling of environments and heterogeneous application of supplemental pollination (see Table 3.3). One set considered only naturally pollinated flowers, with one analysis focused on the two experiments (1 and 2) that involved reciprocal translocation between pink and red environments, whereas the other considered the unilateral component of translocation of red-flowered plants to pink environments in all three experiments. Both analyses included Experiment (1 or 2) and plant Morph (pink or red) as fully crossed, fixed factors and the reciprocal transplant analysis also included Environment (pink or red) crossed with the other factors. The second set of analyses compared naturally and supplementally pollinated flowers to assess pollen limitation. The first analysis in this set involved only data from reciprocal translocation Experiment 2 and assessed the fixed effects of Environment, Morph and Pollination treatment and their interactions. The second analysis considered only data from the pink environment sites of all three translocation experiments and, in addition to the factors included in the parallel analysis in the first set, assessed the fixed effects of pollination treatment (natural or pollen supplemented) and its interactions with the other factors. Plant identity was included as a random factor in the GLMMs for both analyses.

Analysis of inter-morph compatibility included pollination treatment (same population; different population, same environment; or different environment) population nested within environment as crossed, fixed factors.

Results

Pollinator preference and effectiveness

Prosoeca ganglbaueri and *Aeropetes tulbaghia* exhibited strongly contrasting morph preferences when presented with a pair of pink- and red-flowered inflorescences (Fig. 3.1a: Pollinator species $T_1 = 50.31$, $P < 0.001$). *Prosoeca ganglbaueri* significantly preferred flowers of the pink morph at all sites in both regions, regardless of the local flower colour. Specifically, at GG where plants have pink flowers, they first visited pink during 98% of 47 trials, whereas at EH where local plants have red flowers they first visited pink flowers during 89% of 37 trials (pairwise contrast between sites $T_1 = 2.44$, $P = 0.117$, Fig. 3.1a). In contrast, *Aeropetes tulbaghia* mostly first visited the red morph at these sites, with 77 % (LSE = 12, USE = 9) of 9 choices at GG and 96 % (LSE =4, USE = 2) of 44 choices at EH (pairwise contrast between sites $T_1 = 3.94$, $P = 0.104$, Fig. 3.1a). The colour preferences of both *P. ganglbaueri* and *A. tulbaghia* were consistent regardless of the colour of the resident morph of *H. coccinea* at a site (Pollinator x site interaction, $T_1 = 0.06$, $P = 0.939$; Fig. 3.1a). Preference differed between sites ($T_1 = 6.00$, $P = 0.014$): at EH, where the local morph has red flowers, 60% (LSE = 40, USE = 79) of visits were to red flowers, whereas at GG, where the local morph has pink flowers 80% (LSE = 8, USE = 45) of visits were to pink flowers.

The number of pollen grains deposited during single visits depended on the combination of pollinator and floral morph (Pollinator x morph interaction, $G_1 = 20.81$, $P < 0.001$: Fig. 3.1b). In these analyses, which accounted for site effects ($G_1 = 0.194$, $P = 0.66$), pink flowers received significantly more pollen than red flowers during fly visits ($G_1 = 8.86$, $P < 0.02$, Fig. 3.1b). In contrast, red flowers received significantly more pollen than pink flowers during visits by butterflies ($G_1 = 12.41$, $P = 0.05$, Fig. 3.1b).

Common environment experiments

Flowers of plants matching the local morph generally attracted more pollinators than flowers of the foreign morph (site x morph interaction, $G_1 = 15.29$, $P < 0.001$: Fig. 3.2a). At GG, where local plants have pink flowers, translocated pink-flowered plants received

approximately five times more visits per hour than did red-flowered plants ($G_1 = 11.52$, $P = 0.004$, Fig. 3.2a). In contrast, at EH, where plants have red flowers, translocated red-flowered morphs received twice as many visits as did pink morphs, although this difference was not statistically significant ($G_1 = 3.11$ $P = 0.215$, Fig. 3.2a). Overall, the number of visits received by flowers per hour did not differ significantly between sites or morphs (site $G_1 = 1.60$, $P = 0.205$; colour $G_1 = 2.61$, $P = 0.106$: Fig. 3.2a).

Pollen receipt in the common-environment experiments did not differ significantly among the three sites ($G_2 = 0.002$, $P = 0.967$), but was subject to an interaction between morph and environment ($G_1 = 45.81$, $P < 0.001$). Within-environment contrasts showed a consistent advantage of plants that resembled the local morph in both environments ($P < 0.001$ in both cases: Fig. 3.2b).

Translocation experiments

Under natural pollination in the translocation experiments, plants of the local floral morph consistently produced more seeds per flower than did those of the introduced morph (Table 3.4, Fig. 3.3). This was evident for the two reciprocal-transplant experiments (1 and 2) from the significant Morph x Environment interaction (Table 3.4). Specifically, plants of the local pink-flowered morph produced more seeds than those of the introduced red-flowered morph in the pink environment, whereas plants of the local, red-flowered morph outperformed plants of the foreign pink-flowered morph in the red environment (Fig. 3.3a). Similarly, unilateral translocation of red-flowered plants to pink environments in all three experiments resulted in the local pink-flowered plants producing five times more seeds per fruit, on average, than did the foreign red-flowered plants (Table 3.4, Morph effect; Fig. 3.3b). In addition to this home advantage, the reciprocal translocation experiments (1 and 2) involved an experiment x environment interaction (Table 3.4). In particular, plants from the pink environment (all of which were from GG) produced equivalent number of seeds per flower in both experiments, whereas plants from the red environment produced three times more seeds during experiment 2 (EH source) than during experiment 1 (EL source).

Pollen supplementation during translocation experiments revealed pollen limitation of seed production that was stronger for plants of the foreign morph than of the local morph (Table 3.4, Fig. 3.4). For reciprocal-translocation Experiment 2, a significant interaction between the effects of Environment, Morph and Pollination treatment (Table 3.4) resulted because seed set was pollen limited for the foreign morph in each environment (pink in red,

$F_{1,31} = 15.88$, $P < 0.001$; red in pink, $F_{1,31} = 26.14$, $P < 0.001$), but not for the local morph (pink in pink, $F_{1,31} = 2.56$, $P = 0.0687$; red in red, $F_{1,31} = 0.02$, $P = 0.902$: Fig. 3.4a). Comparison of pink sites in all three experiments indicated significant overall pollen limitation for both morphs (pink, $F_{1,52} = 4.93$, $P < 0.05$; red, $F_{1,52} = 6.58$, $P < 0.001$), but the extent of limitation differed between morphs (Table 3.4, Morph x Pollination treatment interaction). In particular, although pink- and red-flowered plants produced equivalent numbers of seeds following supplemental pollination, natural pollination reduced seed production by local pink-flowered plants by half, whereas seed production by foreign red-flowered was 90% less (Fig. 3.4b).

In the greenhouse experiments, seed production of hand-pollinated flowers was not affected by whether the pollen donor was from the same population, a different population from the same environment, or a population of the contrasting environment (Pollination treatment $\chi^2_3 = 3.56$, $P = 0.169$, population within colour morph $\chi^2_3 = 5.14$, $P = 0.399$, Pollination treatment x population within colour morph $\chi^2_3 = 7.84$, $P = 0.166$).

Discussion

Results of this study strongly support the hypothesis that floral variation in *H. coccinea* reflects local adaptation to different locally-dominant pollinator species in the northern and southern Drakensberg regions which are dominated by the pink- and red-flowered morphs, respectively. Contrasting colour preferences of *P. ganglbaueri* and *A. tulbaghia* are evident from their preferential visitation to pink versus red morphs, respectively (Fig. 3.1a). This finding is consistent with the hypothesis of divergence in signalling traits mediated by adaptation to these respective pollinators and explains the more frequent visitation to the pink morph in the northern Drakensberg and to the red morph in southern Drakensberg (Fig. 3.2a). Higher per-visit pollen deposition on pink flowers by flies and on red flowers by butterflies (Fig. 3.1b) suggests that mechanical fit between flower and pollinator has also driven floral morphological divergence. In mixed arrays at sites in both regions, flowers of the local morph were visited more frequently (Fig. 3.2a) and received more pollen (Fig. 3.2b), confirming that pollination environments differ between sites in the northern and southern Drakensberg (Fig. 3.2a) and demonstrating the effect thereof on plant pollination success (Fig. 3.2b). Finally, the seed-set advantage of the local morph under natural pollination (Fig. 3.3) reveals the signature of local adaptation, at least through the pollinator-mediated component of female

success. Although the male component of pollination success was not quantified, greater siring success of local morphs seems likely, given their greater attractiveness to pollinators that were also effective for seed production. Together, these results demonstrate that floral divergence in *H. coccinea* results from local adaptation to pollination by *P. ganglbaueri* in the northern Drakensberg and to *A. tulbaghia* in the southern Drakensberg, driven by differences in the relative abundance of pollinator species and mediated by contrasting signal preferences and mechanical fit of fly and butterfly pollinators.

Before considering the implications of these findings, I address aspects of the implementation of this study that might have introduced experimental artefacts, confounding interpretation of the results. The greater seed set by local than by foreign plants could have resulted because only the latter were excavated, transported and replanted. However, the equivalent seed production following supplemental pollination, regardless of whether plants were local or foreign (Fig. 3.4) is not consistent with this concern. Further, in experiments in which inflorescences of both morphs were transported from their populations of origin to common test sites, pollinator visitation (Fig. 3.1, 3.2a) and pollen receipt (Fig. 3.2b) were consistently higher for local morphs. The combined evidence for similar reproductive potential and higher pollinator attraction and pollen receipt for flowers of the local morph support that a mismatch between pollination environments and floral traits underlies the seed set advantages of plants from the same environment.

Repeated associations between flower colour and effective pollinators, as observed for *H. coccinea* (Chapter 2), suggest a central role of pollinators as selective agents for flower colour (Faegri and van der Pijl 1979, Fenster et al. 2004); however, this "conventional wisdom" has been questioned because of limited evidence linking pollinator colour preferences to differences in reproductive success (Rausher 2008). I previously demonstrated the functional significance of red or pink flowers as cues for visitation by *P. ganglbaueri* and *A. tulbaghia*, respectively, using choice tests with artificial model flowers (Chapter 2). This study additionally demonstrates that this association applies to visitation by these insects in natural *H. coccinea*, regardless of the local flower colour (Fig. 3.2a). Further, visitation by *P. ganglbaueri* to pink-flowered plants and by *A. tulbaghia* to red-flowered plants enhance pollination (Fig. 3.2b) and seed production (Fig. 3.3) more than the opposite combinations. These results decisively link contrasting colour preferences of local pollinators to floral adaptation. The absence of scent in *H. coccinea* (S.D. Johnson, unpublished) and the attraction of pollinators to unscented model flowers suggest that flower colour alone is sufficient to govern contrasting pollinator attraction (see also Bradshaw and Schemske 2003).

Such differential visitation should in turn select for features of floral morphology and orientation that promote pollinator fit (Chapter 2).

Co-variation of intraspecific floral divergence and differences in pollination is often interpreted as evidence for a role of pollinators in driving floral differentiation (e.g. Robertson and Wyatt 1985, Johnson 1997, van der Niet et al. 2014b); although few studies have confirmed that floral trait divergence indeed reflects reciprocal local adaptation to spatial variation in pollination (Herrera et al. 2006, Johnson 2006). Most studies that have quantified local adaptation in such cases have found either a local fitness advantage in only one environment (Boberg et al. 2014) or that one morph outperforms the alternative in both environments (Streisfeld and Kohn 2007, Anderson and Johnson 2009, Sun et al. 2014). In contrast, a pollinator-mediated fitness advantage of the local morph in the contrasting environments of both morphs constitutes evidence that adaptation to *different* pollinators underlies divergence (cf. Kawecki and Ebert 2004). The morphs of *H. coccinea* are thus among the first cases to satisfy the strict criteria for pollination ecotypes (sensu Armbruster 1985, Robertson and Wyatt 1985, see also Johnson 2006). Other relevant cases include inter-population variation of floral morphology of *Erysimum mediohispanicum* in association with differences in generalist pollinator assemblages among sites (Gómez et al. 2009) and of flower colour of *Disa ferruginea* in association with locally conditioned colour preferences of its single pollinator (Newman et al. 2012). *Hesperantha coccinea* differs from these cases in providing evidence of local adaptation of both signal and fit traits caused by a shift between specialised pollinators.

This study evaluated evidence that differences in pollinator assemblages, specifically variation in the relative abundance of the two primary pollinators (Fig. 3.2a) with contrasting colour preferences (Fig. 3.1a), underlies floral divergence among populations of *H. coccinea.* Colour preferences of pollinators can reflect innate bias (Lunau and Maier 1995, Schiestl and Johnson 2013), but conditioning on the colours of local nectar sources can alter preferences of several taxa, including dipteran (Fukushi 1989, Pickens 1990) and nymphalid species (Weiss 1995). Conditioning of local pollinators, especially *P. ganglbaueri* (Whitehead et al. 2019) on the local flower colour could underlie the observed fitness advantage of local floral morphs of *H. coccinea* in their respective environments (Fig. 3.3). However, consistent preferences of *P. ganglbaueri* and *A. tulbaghia* for pink and red, respectively, regardless of the local colour of *H. coccinea* (Fig. 3.1a, see also Chapter 2) confirm that colour preferences of both pollinator species do not reflect conditioning on the local colour of *H. coccinea*. Instead, this association confirms that innate differences in preference and local abundance of

two pollinator species precipitate colour divergence. This conclusion is further supported by the contrasting convergence of pink and red flower colours among other plant species pollinated by *P. ganglbaueri* (Goldblatt and Manning 2000, Anderson and Johnson 2009) and *A. tulbaghia* (Johnson and Bond 1994), respectively (Chapter 2).

Geographic variation in the abundance of different pollinators is generally expected to underlie diversification of animal-pollination systems (Grant and Grant 1965, Stebbins 1970). Adaptation to a novel pollinator occurs due to either qualitative turnover in pollinator species (Johnson 1997, van der Niet et al. 2014b, Streinzer et al. 2019) or quantitative variation in relative pollinator abundance (Robertson and Wyatt 1985, Waser 2001, Gómez et al. 2009). In the case of *H. coccinea*, both may contribute to the geographic differences in pollinator availability that underlie the reproductive advantage of the local morph observed in this study (Fig. 3.3). Although the two pollinator species of *H. coccinea* have overlapping distributions (Chapter 2), *Prosoeca ganglbaueri* occurs more frequently at high elevation and is not recorded below 1100 m.a.s.l., whereas *Aeropetes tulbaghia* occurs at lower elevations. Thus, flies are absent from low elevation sites, but likely more abundant at high sites.

Correspondingly, *H. coccinea* populations with pink flowers tend to occur more frequently at higher elevation sites, whereas populations with red flowers are more common at lower elevation sites (Chapter 2). At mid elevation, mixed sites, sites with pink-flowered morphs and sites with red-flowered morph occur. In this study, two of the source populations with red flowers occur at similar elevation to a pink-flowered experimental site (Table 3.1). Relative rates of visitation to paired morphs in arrays at these sites (Fig. 3.2a) suggest that differences among populations in which pollinator is most commonly observed (Chapter 2) and the advantage of plants of the local flower colour morph reflect a spatial quantitative mosaic of pollinator availability in the mid-elevation study sites. Diverse factors including host plant distributions (Johnson 1997), fine-scale topography (Johnson and Bond 1992) and biotic interactions (Waser 1983), may influence local pollinator abundance; which determine the geographic mosaic of pollinator availability and adaptation of *H. coccinea* to different pollinator at mid-elevation sites remain to be confirmed.

Heterospecific pollen receipt is expected to play a major role in floral evolution, including pollinator adaptation (Moreira-Hernández and Muchhala 2019), and is particularly prevalent at mid to low elevations (Arceo-Gómez et al. 2019). In the Drakensberg region, *H. coccinea* is one of more than 20 species, including several congeners that depend on *P. ganglbaueri* for pollination (Goldblatt and Manning 2000, Anderson and Johnson 2009). Therefore, competition for fly pollination might promote local adaptation to butterfly

pollination in *H. coccinea*, especially at elevations where both pollinators occur (Chapter 2). Lower visitation (Fig. 3.2a) and the reproductive disadvantage of the pink morph in red environments at intermediate elevation (Fig. 3.3a) may result from rare and inefficient visitation by locally-dominant butterflies and ineffective visitation by local flies. Flies were observed less frequently than butterflies at site EH and visited flowers of several other plant species, including another *Hesperantha* species, and they carried visible foreign pollen. In contrast, butterflies visited *H. coccinea* almost exclusively at that site. Pollinator sharing can affect both visitation frequency and effectiveness (Robertson 1895, Waser 1978, Morales and Traveset 2008, Mitchell et al. 2009, Balfour et al. 2015) and the reproductive costs are especially severe among sympatric congeners that share a pollinator (Waser 1983). If competition among plant species for fly pollination affects the availability and or effectiveness of fly pollinators for *H. coccinea* where both pollinators occur, local adaptation to butterfly pollination in *H. coccinea* may also reflect character displacement (Armbruster 1985, Levin 1985, Fishman and Wyatt 1999, Grossenbacher and Stanton 2014).

This study revealed strong evidence that local adaptation to contrasting pollinators underlies divergence into distinct floral morphs in *H. coccinea*; however, a few intermediate and mixed populations also occur in the central Drakensberg (Chapter 2). Strongly contrasting pollination environments and extensive geographic separation between populations of contrasting morphs increase the likelihood of local adaptation by minimising counteracting effects, such as maladaptive gene flow (Schluter 2000, Hereford 2009). A deeper understanding of the counteracting effects of pollinator adaptation and gene flow in shaping floral variation throughout the distribution of *H. coccinea* could be gained from population genetic analyses (preliminary results using SSR markers show strong genetic differentiation among floral morphs: unpublished results). At the northern extremity of the distribution of *H. coccinea*, populations of both red and pink morphs also occur, with pink-flowered populations at higher elevation sites, providing an opportunity to investigate whether replicated intraspecific pollinator-mediated divergence has followed parallel evolutionary trajectories, and whether shifts between morphs are reversible. Translocations along elevation gradients (Clausen et al. 1940, Sun et al. 2014), encompassing both intermediate elevation sites and sites at low elevations where *P. ganglbaueri* does not occur (Hargreaves et al. 2014), and including quantification of heterospecific pollen transfer (Ashman et al. 2020) would be useful to investigate how qualitative turnover in pollinator species, quantitative variation in relative pollinator abundance and competition interact to

determine adaptation to different pollinators and the current distribution of colour morphs *H. coccinea*.

Tables

Table 3.1 Co-ordinates, flower colour and dominant flower visitor of *Hesperantha coccinea* populations used for reciprocal translocation experiments. Pollinator data are based on observations during five flowering seasons (see Chapter 2 for details).

Region	Flower colour	Population	Most frequent pollinator	Location		Elevation (m.a.s.l.)
Northern Drakensberg	Pink	Mahai (MH)	*P. ganglbaueri*	-28.695S	28.906E	2047
	Pink	Golden Gate (GG)	*P. ganglbaueri*	-28.507S	28.620E	1931
Southern Drakensberg	Red	Elands Heights (EH)	*A. tulbaghia*	-30.818S	28.207E	1759
	Red	Bushman's Nek (BN)	*A. tulbaghia*	-29.843S	29.209E	1765
	Red	Elliot (EL)	*A. tulbaghia*	-31.313S	27.867E	1490

Table 3.2 Sampling information (number of inflorescences in round brackets, number of flowers in square brackets) for common environment experiments assessing pollinator preference, single-visit pollen deposition and total pollen receipt. Each trial used pink- and red-flowered inflorescences from sites other than the local *H. coccinea* population in which they were placed.

Response	Context site (environment)	Pink source	Red source
Pollinator behaviour	GG (pink)	MH (8)	EL (8)
	EH (red)	MH (17)	EL (17)
Single-visit deposition	GG (pink)	MH [32]	EL [11]
	EH (red)	MH [14]	EL [11]
Pollen receipt	GG (pink)	MH (20) [40]	EL (18) [36]
	MH (pink)	GG (11) [22]	BN (11) [23]
	EL (red)	MH (16) [29]	EH (16) [20]

Table 3.3 Numbers of plants and flowers (in parentheses) sampled for three translocation experiments assessing seed production following natural pollination and supplemental hand-pollination. Population environment (red or pink) is given in parentheses below site of origin.

		Experiment 1		Experiment 2		Experiment 3	
		Site GG	Site EL	Site GG	Site EH	Site MH	Site BN
Plant status	Pollination	(pink)	(red)	(pink)	(red)	(pink)	(red)
Local	Natural	12 (63)	16 (89)	9 (36)	11 (62)	10 (37)	-
	Supplemented	12 (12)		9 (9)	8 (8)	10 (10)	
Foreign	Natural	11 (60)	11 (65)	10 (39)	12 (61)	7 (25)	-
	Supplemented	11 (11)		10 (10)	8 (8)	7 (7)	

Table 3.4 Results of generalised linear models of effects on seed production per flower following natural pollination or supplemental outcross pollen to alleviate any pollen limitation. Main effects include Experiment (1, 2 or 3), context Environment (Pink i.e. Northern Drakensberg) or (Red i.e. Southern Drakensberg), plant morph (Pink or Red) and Pollination treatments (Natural or Supplemental cross-pollen). Separate analyses are provided for particular experiments or environments, owing to the sampling design. All analyses accounted to measurement of multiple flowers per plant.

	Natural pollination		Supplemental pollination	
Effect	Exp 1 and 2	Pink Env	Exp 2	Pink Env
Experiment (Exp)	$G_1 = 6.75$**	$G_2 = 0.04$		$G_2 = 0.02$
Environment (Env)	$G_1 = 0.03$		$G_1 = 0.81$	
Exp x Env	$G_1 = 9.73$**			
Morph	$G_1 = 2.98$	$G_1 = 24.48$***	$G_1 = 0.07$	$G_1 = 11.75$***
Morph x Exp	$G_1 = 2.45$	$G_2 = 2.80$		$G_2 = 1.05$
Morph x Env	$G_1 = 45.13$***		$G_1 = 12.93$***	
Morph x Exp x Env	$G_1 = 0.30$			
Pollination (Poll)			$G_1 = 28.43$***	$G_1 = 39.95$***
Poll x Exp				$G_2 = 0.01$
Poll x Env			$G_1 = 2.43$	
Poll x Morph			$G_1 = 0.19$	$G_1 = 12.89$***
Poll x Exp x Morph				$G_2 = 0.73$
Poll x Env x Morph			$G_1 = 14.28$***	

** $P < 0.01$, *** $P < 0.001$

Figures

Figure 3.1

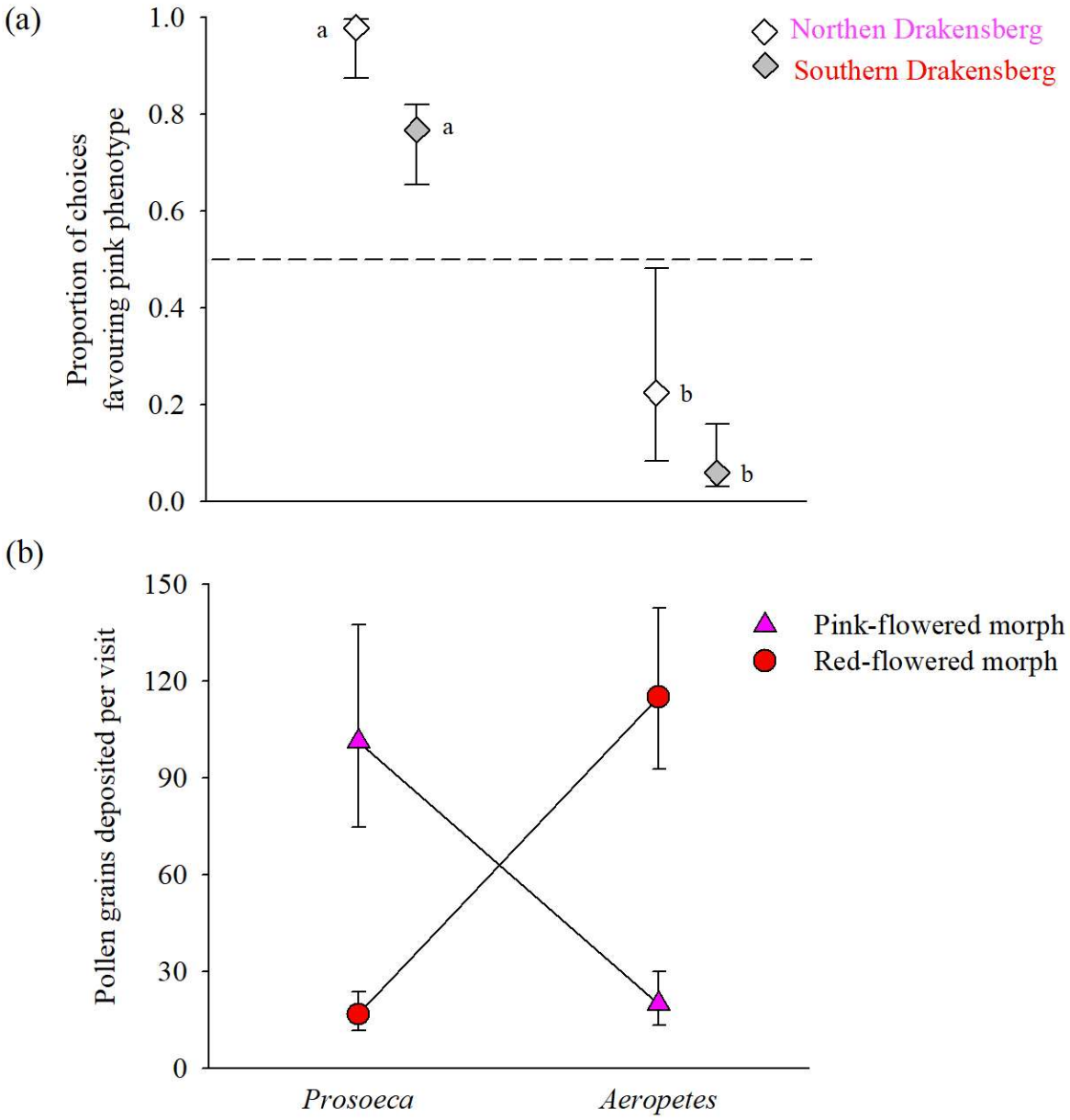

Fig. 3.1 Mean (a) preference during choice trials (± 95% CI) and (b) pollination effectiveness (± SE) of *P. ganglbaueri* and *A. tulbaghia* for pink- and red-flowered morphs of *H. coccinea* in a pink-flowered site (GG) in the northern Drakensberg and a red-flowered site in the southern Drakensberg (EH) site. (a) Proportion of first visits to flowers of the pink morph when offered a pair of inflorescences of both morphs at GG (open diamonds) and EH (filled diamonds). Contrasting letters indicate significantly different preferences ($P < 0.05$). (b) Numbers of pollen grains deposited per visit on stigmas of pink (triangles) and red (circles) flowers of *H. coccinea.* See text for statistical results.

Figure 3.2

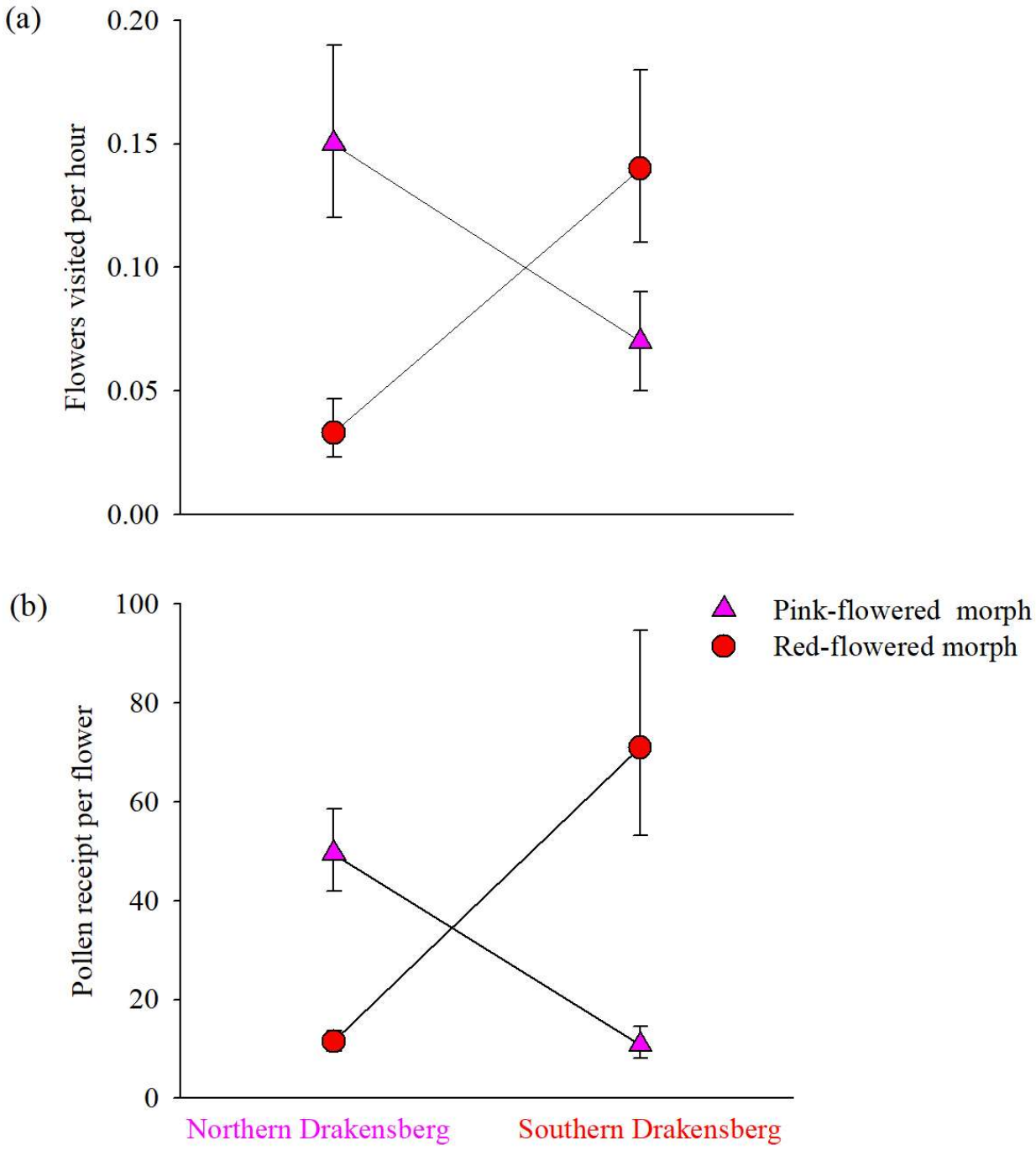

Fig. 3.2 Mean (± SE) (a) visits per hour and (b) pollen receipt per flower for pink- and red-flowered morphs of *Hesperantha coccinea* in pink- and red-flowered populations, respectively. See text for statistical results.

Figure 3.3

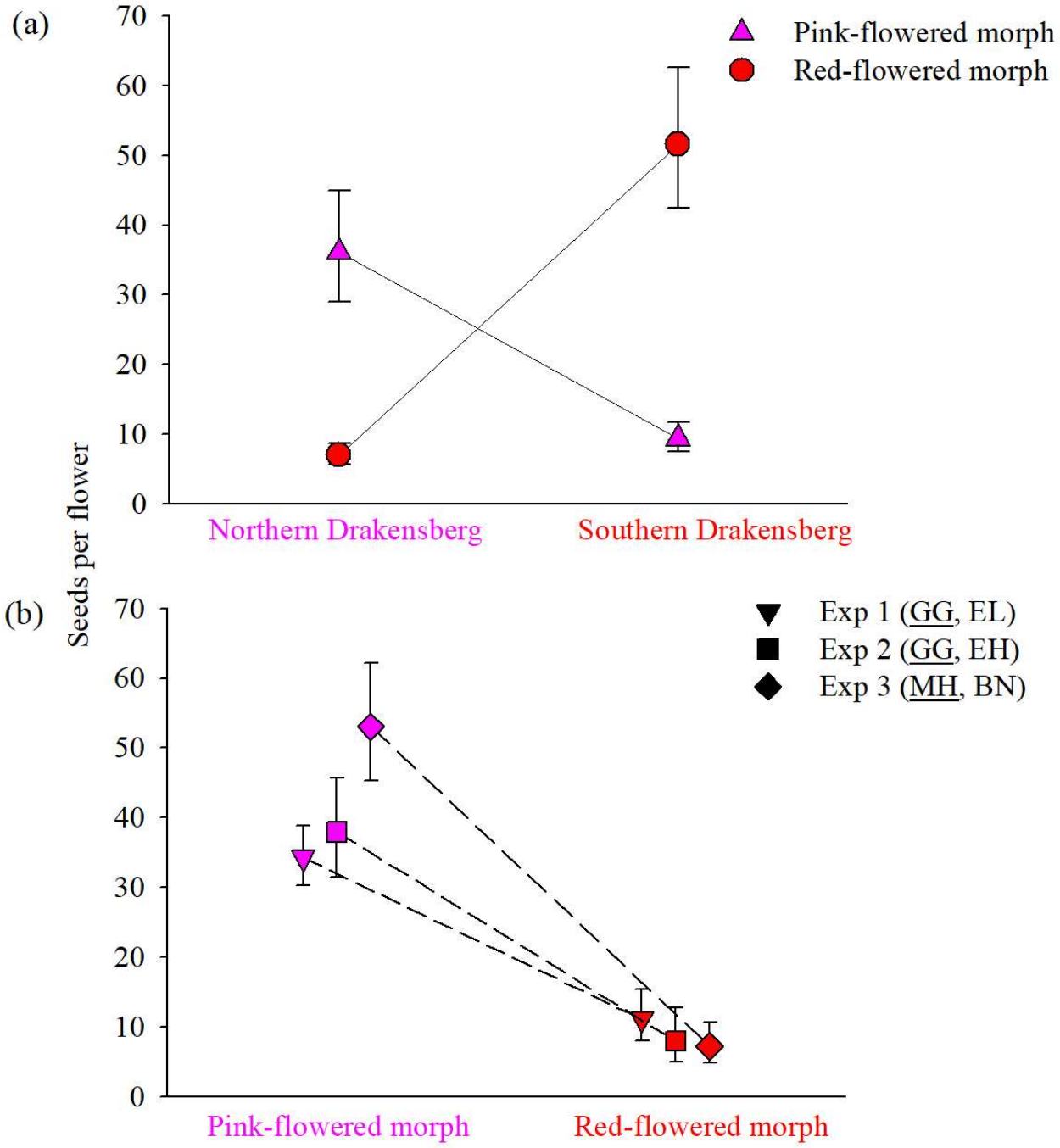

Fig. 3.3 Mean (± SE) seed production per flower of pink- and red-flowered morphs of *Hesperantha coccinea* in (a) pink- and red-flowered populations of Experiment 1 and 2 and (b) pink-flowered populations of all three experiments. Underlined site codes indicate the sites where experiments were conducted.

Figure 3.4

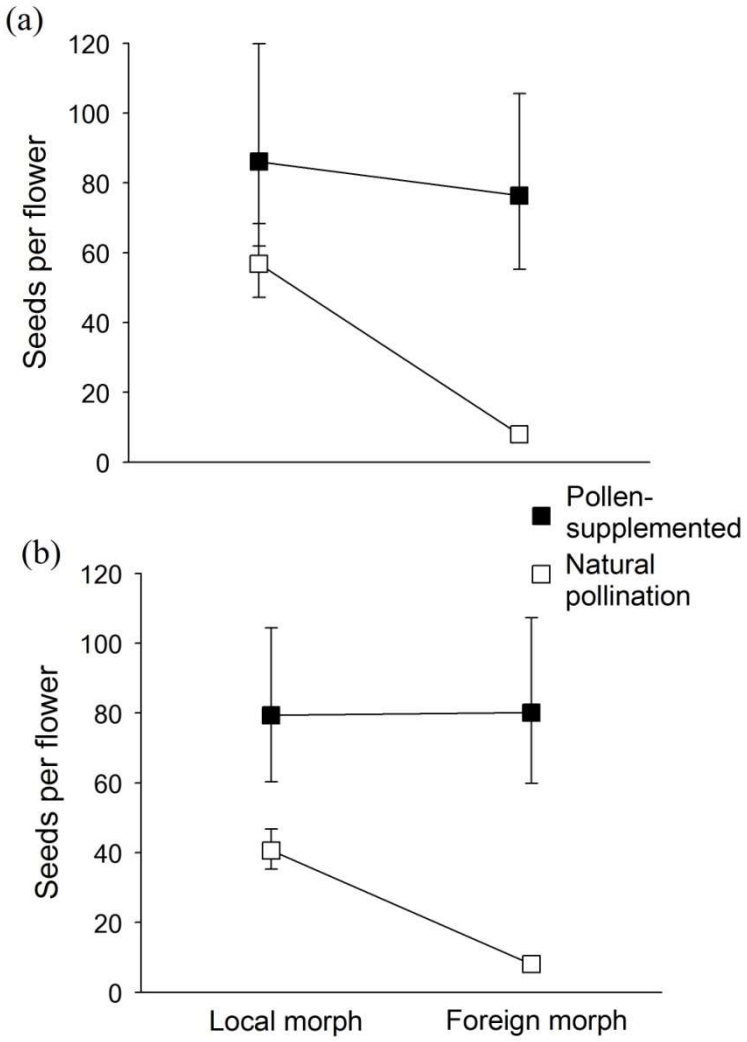

Fig. 3.4 Mean (± SE) seed production per flower of naturally pollinated and pollen-supplemented flowers on plants of local and foreign morphs of *Hesperantha coccinea* in (a) the pink- and red-flowered populations of Experiment 2 and (b) pink-flowered populations of all three experiments.

Appendix

Table A3.1 Numbers of flowers hand-pollinated to assess compatibility within and between populations and environments.

	Environment			
	Maternal plant origin			
	Pink		Red	
Pollen origin	GG	MH	BN	EL
Within population	10	6	16	5
Between populations within environment	6	5	11	4
Between environments	8	3	11	4

CHAPTER 4: “BEST-OF-BOTH-WORLDS” REPRODUCTION IN A SHOWY SOUTHERN AFRICAN IRIS: MIXED MATING, DELAYED AUTOFERTILITY AND WEAK INBREEDING DEPRESSION.

Abstract

The evolution of self-fertilisation and shifts to a novel pollinator are generally considered separate evolutionary phenomena, however, recent theory suggests that delayed selfing may facilitate pollinator shifts. The timing and effectiveness of self-pollination and the relative fitness of selfed offspring are key factors affecting the potential for a pollinator shift. "Best-of-Both-Worlds" (BOBW) reproductive systems which combine specialised pollination and delayed autofertility represent ideal candidates for such shifts. Pink- and red-flowered ecotypes of the southern African iris *Hesperantha coccinea* are specialised for pollination by long-proboscid flies and butterflies, respectively; however, preliminary evidence suggests that this species can also reproduce independently of pollinators. To assess potential for BOBW reproduction and determine whether the pollinator shift has occurred in spite of autofertility in *H. coccinea* I used controlled pollinations to investigate self-compatibility, autofertility, the timing of autonomous self-pollination and inbreeding depression, and SSR's to quantify mating patterns. In two populations of each ecotype, self- and outcross pollination resulted in equivalent seed set. Plants from all populations could reproduce independently of mates or pollinators, with autofertility indices ranging from 0.54 to 0.9. In a pollinator-free greenhouse, emasculation of flowers during different stages of anthesis demonstrated that autonomous self-pollination occurs mostly late during floral lifespan. Performance of selfed and outcrossed progeny was similar for five of six measured life-stages, consistent with limited cumulative inbreeding depression (0.1 to 0.25 for the three studied populations). Outcrossing rates estimated using eight SSR markers confirmed that populations of both ecotypes exhibit mixed mating (multi-locus estimates = 0.37 and 0.67). These results provide evidence consistent with "Best-of-Both-Worlds" reproduction in both ecotypes of *H. coccinea*. In addition, weak inbreeding depression in both pollinator-specialised ecotypes suggests that the fraction of cross pollen received by stigmas is unlikely to explain pollinator shifts and this points to siring success as a key driver of pollinator specialisation in "Best-of-Both-Worlds" reproduction.

Key words: Autofertility, self-compatibility, mating system, inbreeding depression, *Hesperantha coccinea*, Iridaceae.

Introduction

Two major trends in the evolution of plant reproductive systems - the evolution of autofertility and transitions to a novel pollinator - are widely considered to be driven by selection to alleviate pollen limitation and to have contrasting evolutionary consequences. Firstly, autofertility alleviates pollen limitation of female reproductive success and thereby the proximate selective conditions for female fitness that could drive future pollinator-shifts (Larsen and Brett 2000, Knight et al. 2005; also see Ashman and Morgan 2004, Teixido and Aizen 2019). Further, selfing affects the floral traits that could attract a novel pollinator: Even within a few generations (Bodbyl Roels and Kelly 2011) increased reliance on selfing is associated with the evolution of a selfing syndrome of floral traits (Sicard and Lenhard 2011), including reduced investment in pollinator attraction and reward. Finally, repeated autogamous reproduction results in reduced genetic variation (Charlesworth and Charlesworth 1995), associated adaptive potential (Stebbins 1957, Glemin and Ronfort 2013) and diversification rates (Takebayashi and Morrell 2001, Goldberg et al. 2010). Thus, although the evolution of selfing can provide a reproductive assurance benefit in the short-term, shifts to predominant selfing are often considered to represent a long-term evolutionary dead-end (Stebbins 1957, Igic and Busch 2013, Wright et al. 2013, Cheptou 2019). In contrast, when pollen limitation is alleviated by a shift to a novel pollinator, reliance on outcrossing, associated genetic diversity, maintenance of floral investment in pollinator attraction and thus potential for future pollen limitation and pollinator shifts are retained. For these reasons, pollinator shifts and shifts to selfing could be considered alternative evolutionary responses to pollen limitation (e.g. Barrett 2002, Johnson 2006, Harder and Aizen 2010, van der Niet and Johnson 2012, Opedal 2019).

The prevalence of "showy selfers" that invest in attractive, rewarding flowers characteristic of pollinator-mediated outcrossing and yet are highly autofertile (Darwin 1862, Willmer 2011), seemingly contradicts the idea that selfing and pollinator adaptation represent alternative evolutionary trajectories. A survey of 80 species from 38 families found that 80% of autofertile species also have specialised animal-pollination systems (Fenster and Martén-Rodríguez 2007). The "Best-of-Both-Worlds" (BOBW) hypothesis suggests the combination of delayed autofertility and specialised pollination exhibited by showy selfers is an optimal strategy (Fenster and Martén-Rodríguez 2007, Goodwillie and Weber 2018). A logical but largely untested extension of this idea is that the same conditions that maintain specialised pollination in BOBW systems could facilitate adaptive shifts to novel pollinators in

autofertile species (also see Wessinger and Kelly 2018). The mode of selfing and magnitude of inbreeding depression are thought to be key for maintenance of BOBW systems. When autonomous selfing only occurs after opportunities for outcrossing have passed, potential outcrossing is maximised whenever possible, and autofertility provides reproductive assurance when it is not (Lloyd 1992, Goodwillie et al. 2005, Fenster and Martén-Rodríguez 2007). Empirical surveys confirm that autofertility in BOBW species commonly involves delayed self-pollination, which is frequently facilitated by mechanical reduction of anther-stigma separation during floral life (Fenster and Martén-Rodríguez 2007, Goodwillie and Weber 2018). Experimental work has confirmed that outcrossing rates of BOBW species reflect variation in the magnitude of pollinator failure and the corresponding reproductive assurance benefit of autonomous self-pollination (e.g. Kalisz et al. 2004, Koski et al. 2019). BOBW systems are also expected to be characterised by substantial inbreeding depression, such that benefits of outcrossing and adaptations for pollinator mediated reproduction are maintained (Goodwillie and Weber 2018). However, the relatively few studies of inbreeding depression for BOBW species have reported both strong (Herlihy and Eckert 2002, Ruan et al. 2011) and weak inbreeding depression (e.g. Kalisz et al. 2004, reviewed in Goodwillie and Weber 2018). An alternative mechanism for adaptive maintenance of pollinator specialisation in showy selfers involves selection through fitness components other than female outcrossing (Fenster and Martén-Rodríguez 2007), but empirical evidence for this is lacking. Thus, whether high inbreeding, or other factors underlie the maintenance of pollinator specialisation in BOBW species remains to be determined.

Whether adaptation to (different) pollinators can be maintained in BOBW species depends on whether the associated mixed mating can be an evolutionary stable strategy, or, as has often been contended, represents a transitional stage between predominant outcrossing and selfing. Most theory that predicts exclusive, strict selfing or outcrossing focusses on whether costs of selfing, primarily, inbreeding depression counter associated automatic selection advantage and reproductive assurance benefits (Lande and Schemske 1985, Charlesworth and Charlesworth 1987). In contrast, theory that emphasizes aspects of pollination ecology, including the heterogenous consequences of different modes of selfing (Lloyd 1979, Lloyd and Schoen 1992) and features of the pollination environment and pollination processes predicts that mixed mating can be stable, including at various magnitudes of inbreeding depression (reviewed by Goodwillie et al. 2005, also see Johnston 1998, Johnston et al. 2009). Generally similar inbreeding depression of mixed mating taxa and those that outcross obligately (Winn et al. 2011), also suggests, contrary to Fisher (1941),

that inbreeding depression is not the key determinant of mating system evolution. Further, models that incorporate potential for inbreeding depression to evolve in response to mating (Lande and Schemske 1985) generally predict that mixed mating can be stable under a wider variety of conditions, including when inbreeding depression is relatively weak (see Goodwillie et al. 2005, Eckert et al. 2006). Among these, are the models of Johnston (2009) which propose that correlations between fitness components can maintain mixed mating are particularly relevant to showy selfers, because delayed selfing inherently involves functional relationships between pollinator mediated and autonomous contributions to mating. Specifically, in showy selfers, pollinator-mediated seed production and siring success are expected to positively correlated, and both should be inversely related to seed production through autonomous selfing. Johnston (2009) predicts that relationships consistent with these correlations can result in stable mixed mating, including in association with varying magnitudes of inbreeding depression (Figures 5E in Johnston 2009). Thus, when effects of pollination ecology on the evolution of mating systems are considered, it appears that strong inbreeding depression should not necessarily be expected in BOBW systems, and that BOBW mixed mating can at least in theory be sufficiently stable to enable the same conditions that allow maintenance of pollinator adaptation to facilitate a shift to a novel pollinator.

Recent theoretical modelling by (Wessinger and Kelly 2018)specifically addressed whether selfing may enable pollinator shifts and found that delayed selfing in particular promotes shifts to a less frequent but more efficient pollinator, through two mechanisms. Firstly, the reproductive assurance provided by delayed autonomous selfing enables shifts to a novel pollinator even when that pollinator is rare. Secondly, reproduction via selfing provides an additional possibility for fixation of advantageous alleles, by allowing recessive alleles to bypass Haldane's sieve. Importantly, models predict that increased efficiency of delayed selfing and thus also by increased relative fitness of selfed offspring broaden conditions under which a pollinator shift is favoured, even when reproductive success is pollen limited. By these mechanisms, delayed selfing may increase evolutionary lability and provide an important alternative pathway contributing to the significant signature of pollinator-driven divergence in angiosperms (Johnson 2006, Kay and Sargent 2009, van der Niet and Johnson 2012). In support of the associated prediction that shifts towards more efficient pollination systems, in particular systems associated with loss of function traits should be more common in self-compatible lineages, Wessinger and Kelly (2018) found that self-compatibility was significantly more strongly associated with (putatively derived) hummingbird pollination systems than in angiosperms in general, and, within lineages, in

relation to species characterised by less efficient bee pollination. However, whether this association reflects a tendency of hummingbird pollinated species to evolve self-compatibility (Fenster et al. 2004) or vice versa cannot be determined form the existing data (Wessinger and Kelly 2018). Thus, confirmation of the idea that selfing can facilitate a pollinator shift, awaits more detailed empirical studies.

I identified *Hesperantha coccinea* (Backh. and Harv.) Goldblatt and J.C. Manning (Iridaceae) as an ideal study system to determine whether BOBW reproduction in a showy selfer is associated with adaptation to a novel pollinator. *Hesperantha coccinea* produces showy red or pink flowers with floral tubes up to 4 cm long that produce ample nectar (approx. 4µl per flower; Goldblatt 2003, Chapter 2). Differences in flower colour among populations are associated with differences in floral morphology, orientation and nectar properties, and with differences in pollination: populations of pink-flowered plants typically occupy higher sites in the northern Drakensberg region of eastern South Africa, and are pollinated almost exclusively by the long-proboscid fly *Prosoeca ganglbaueri*. Red-flowered populations occur more widely, including over a broader elevational distribution, ranging from the Amatole mountains in the south to the northern Drakensberg region, and are pollinated by large butterflies, primarily *Aeropetes tulbaghia* (chapter 2). However, plants conforming to both ecotypes have become naturalised on other continents and isolated *H. coccinea* plants in gardens produce viable seeds in the absence of mates (Wolff et al. 2009). This combination of specialised pollination and autofertility suggests that *H. coccinea* is a showy selfer in which a pollinator shift has occurred. If the two ecotypes both exhibit characteristics of BOBW reproduction, autofertility was likely an ancestral trait in *H. coccinea,* and the pollinator shift occurred in spite of autofertility. In this chapter, I use controlled pollinations and SSR's to assess whether traits of populations of both pollination ecotypes of *H. coccinea* are consistent with BOBW reproduction. Specifically, I assess evidence for 1) self-compatibility, 2) autofertility, 3) timing of self-pollination, 4) the extent of inbreeding depression and 5) mixed mating in populations of both ecotypes.

Materials and Methods

Study species and sites

Hesperantha coccinea is a perennial geophyte that grows along montane watercourses from the Drakensberg escarpment in the eastern cape of South Africa to the highlands of eastern

Zimbabwe (Goldblatt and Manning 1996b). plants flower from December to April, depending on location, and bear 6-15 large, showy flowers on one or more inflorescences (Goldblatt 1993). individual flowers last from three to four days and close overnight between c. 18h00 and 07h00 (Goldblatt et al. 2004).

Like other H*esperantha* species, *H. coccinea* flowers are weakly protandrous (Goldblatt et al. 2004), with the inward-facing anthers dehiscing soon after flowers first open. Nevertheless, stigmas also become receptive during the first day of anthesis (indicated by longitudinal unfurling of the stigma lobes, exposing the stigmatic papillae), so pollen receipt and removal can occur simultaneously during pollinator visits. both the stamens and style branches are elongate and spread laxly, diverging at the mouth of the floral tube (Goldblatt and Manning 1996b), with anthers and stigma branches initially separated by up to 20 mm. however, herkogamy declines during anthesis, so that stigmas frequently contact anthers during the third day of floral life (R. Cozien, personal observations).

Breeding system

The breeding system of *H. coccinea* was assessed with controlled pollination experiments using plants grown from seeds collected from two pink- and two red-flowered populations (Table 1). These plants were maintained in a pollinator-free greenhouse at the Botanical Gardens of the University of KwaZulu-Natal (Pietermaritzburg Campus). Experiments involved 174 flowers on 54 plants (Table A4.1).

During March and April 2012, one to six flowers on each of 11 to 21 individuals per population were assigned randomly to one of four pollination treatments. To assess self-compatibility, stigmas of emasculated flowers were pollinated with pollen from the same flower or another flower on the same inflorescence ("self" treatment), or with pollen from up to three flowers from different plants ("cross" treatment). To assess capacity for autonomous seed production (autofertility), intact flowers that could self-pollinate autonomously were either left unmanipulated ("unmanipulated" treatment) or supplementally pollinated with cross pollen ("supp" treatment). To prevent autonomous self-pollination for the self and cross treatments, anthers were removed from flowers before they dehisced. For hand-pollination treatments, pollen was applied to receptive stigmas during the first day of anthesis using anthers held in forceps. Stigmas were considered receptive if their branches had unfurled, papillae were visible and pollen adhered readily. The receptivity of stigmas exhibiting these characteristics was confirmed in a separate experiment using the peroxidase test (Dafni

1992). Treated flowers were marked with jeweller's tags attached to the floral tube. Three to four weeks after experimental flowers wilted, fruit development was recorded and ovules and developed seeds were counted for each developed fruit.

For each treatment I calculated an index of self-incompatibility (ISI) and an index of autofertility (IAF) for each population. The ISI was calculated as

$$ISI = 1 - \frac{S_s}{S_x},$$

where S_s and S_x are mean seeds per ovule following hand self- and cross-pollination, respectively (Lloyd 1965, Raduski et al. 2012). ISI = 0 indicates equivalent fecundity of selfed and outcrossed flowers, whereas ISI = 1 indicates complete dependence on cross-pollen for seed production. The IAF was calculated as

$$IAF = \frac{S_u}{S_+},$$

where S_u and S_+ are mean seed set by unmanipulated flowers and those that received supplemental cross-pollen, respectively (Lloyd and Schoen 1992). AFI = 0 indicates complete dependence of seed production on vector-mediated pollination.

Mechanism and timing of autonomous self-pollination

I assessed the characteristics of autonomous self-pollination in two ways. First, to document the dynamics of stigma-anther separation (herkogamy), I measured the distance between the tip of each anther and the closest stigma branch on three consecutive days of anthesis. One flower was measured on each of eight plants using digital callipers. Second, to identify when autonomous deposition of self-pollen occurs during a flower's life pollen deposition and seed set were compared for flowers that were emasculated 24 h or 48 h after anthesis, or were not emasculated. This experiment was conducted in a pollinator-free greenhouse during February 2012 using plants grown from seeds collected from the Kamberg population. The treatments were applied to two groups of plants to separately assess the effects of pre-emasculation period on self-pollen deposition on stigmas (40 plants, 63 flowers) and seed production (10 plants, 38 flowers). To quantify pollen deposition, stigmas were collected when experimental flowers had begun to wilt on the fourth morning after anthesis and mounted in fuchsin glycerine jelly (Beattie 1971). Pollen grains were counted at 40x under a compound microscope (Kearns and Inouye 1993). To assess fecundity, stigmas were left intact and fruits were allowed to develop for three weeks, when their developed seeds were counted.

Inbreeding depression

I quantified inbreeding depression based on the relative performance of selfed and outcrossed progeny during *in situ* seed development and *ex situ* seed germination, plant growth and flowering for three populations. For Bushman's Nek (BN: red-flowered) plants, flowers were pollinated during March 2009 and seeds were sown during June 2009. For Elliot (EL: red-flowered) and Kamberg (KM: pink-flowered) plants, flowers were pollinated during March 2011 and sown during May 2011. For further details of populations and sampling see Table S2.

Controlled hand-pollination was used to compare *in situ* seed production following selfing and outcrossing and to produce seeds for the *ex situ* study. In each population, inflorescences of 7 to 25 plants were bagged before anthesis with 2-mm diameter mesh bags to exclude pollinators. Two flowers on each inflorescence were emasculated during bud stage and pollinated after they opened, as described above for self- and cross-pollination treatments. Pollen for the cross-pollination treatment was collected from one to three donor plants at least 5 m from each recipient plant. Four weeks later, fruit development was recorded, mature fruits were collected and the developed seeds were counted. Seeds were not counted for fruits that had begun to dehisce or showed evidence of predation.

To quantify the relative success of selfed and outcrossed progeny, 30 seeds from each fruit (henceforth, a "Seed family") were sown in potting soil in 15-cm diameter pots in a greenhouse at the Botanical Gardens of the University of KwaZulu-Natal (Pietermaritzburg). All plants were transplanted into 30-cm diameter pots three months after sowing. Plants from BN seeds were maintained in the greenhouse throughout the experiment. Owing to limited greenhouse space, plants from EL and KM seeds were moved into a shadehouse, where plants were maintained for an additional 12 months (December 2012). Germination rate (number of germinated seeds of the 30 sown) was recorded one month after sowing. The length of the longest leaf of the tallest plant in each seed family was recorded 3 months after sowing to compare success at seedling stage, and after 18 months to compare height at maturity (plants began producing flowers after approximately 18 months). Finally, between February and April 2010 (BN) and 2012 (KM and EL) the inflorescences and flowers (including buds, wilted and open flowers) were counted for up to five plants per seed family. Inbreeding depression was calculated for life-cycle stage *i* as

$$\delta_i = 1 - \frac{\bar{M}_{s,i}}{\bar{M}_{x,i}},$$

where $\bar{M}_s$ and $\bar{M}_x$ are mean performance by selfed and outcrossed offspring, respectively. Cumulative inbreeding depression for each population was calculated as

$$\delta = 1 - \prod \frac{\bar{M}_{s,i}}{\bar{M}_{x,i}}$$

(Husband and Schemske 1996).

Mating system

I estimated female outcrossing rates for populations EL (red form) and GG (pink form) using nine microsatellite loci previously developed for *H. coccinea* (Wolff et al. 2009) that were combined into two multiplex reactions (for details see Appendix A4 and Table A4.3). Maternal leaf tissue and one mature fruit were collected from each of 17 and 20 maternal plants from EL and GG, respectively, during March 2011. Thirty seeds from each fruit were sown as described above for the inbreeding depression experiment. Two months after sowing, approximately 1 cm^2 of leaf material was harvested from six to eight seedlings (mean ± SD = 7.94 ± 0.22) from each seed family. Assessment of mating outcomes based on seedling tissue is unlikely to bias estimates of outcrossing for *H. coccinea*, as comparisons of relative seed development, germination and seedling growth (Fig. 4.3 and Table 4.1) indicate little effect of inbreeding on survival from zygote to seedling. DNA was extracted from silica dried leaf material using the Qiagen (Valencia, CA, USA) DNeasy Plant Mini Kit. Fragment analysis was conducted with an Applied Biosystems ABI3130xl Genetic Analyser and all samples were genotyped using GeneMarker v2.2.0 (SoftGenetics, LLC^{TM}). Fragment-length bins were automatically assigned for all alleles observed in maternal samples and manually checked prior to automatic scoring of all samples. Twelve samples were amplified and genotyped twice, with no differences in scored genotypes. All 342 sample genotypes were rescored twice, revealing an average scoring error rate across loci of 1.8 ± 0.6%. Mating system parameters, including the multilocus outcrossing rate, t_m, correlation of paternity r_p, correlation of selfing among loci, r_s, and parental inbreeding coefficient, F_{IS}, were estimated using maximum likelihood methods in MLTR (Ritland 2002). Sampling error estimation was based on 1000 bootstraps and resampling of seed families. Known maternal genotypes were included in the analysis and pollen and ovule frequencies were constrained to be equal.

Statistical analyses

Statistical analyses involved generalised linear models (GLM) (McCullagh and Nelder 1989) as implemented in SPSS 26 (IBM Inc.). Count data, including pollen grains, fruits, flowers, and inflorescences, were analysed with Poisson or negative binomial distributions, according to model fit, and the ln link function. Plant height was analysed with a Gaussian distribution and identity link. Proportion data (seeds per ovule and germinated seeds per sown seeds) were analysed with a binomial distribution and logit link function, with likelihood estimation of the mean-variance adjustment parameter. Ovules could not be counted for 8 of 174 flowers in the breeding-system experiment because fruits failed to develop, so they were assigned the population average ovule number for relevant statistical analysis. Analyses of breeding system tested the fixed effects of pollination treatment, colour and population nested within flower colour and their interactions. Analyses of inbreeding depression included only population and treatment as crossed fixed factors, because this experiment involved two red populations, but only one pink-flowered population. To compare adult height independent of previous life stages, analysis of mature plant height included seedling height for each seedling family as a ln-transformed covariate.

Generalised estimating equations (GEE: Liang and Zeger 1986) were used to account for correlated responses in analyses that involved multiple measurements per plant. An exchangeable correlation matrix was used in analyses of breeding system and inbreeding depression, whereas an autoregressive correlation matrix was used to analyse the timing of self-pollination. In all GEE analyses, statistical inference involved Wald χ^2 tests.

Significant effects and interactions were explored further using multiple comparisons, with sequential Šidák correction. All plotted means and standard errors are back-transformed from the scale of analysis.

Results

Breeding system

Responses to different pollination treatments indicate breeding system differences for the proportion of ovules setting seed (Fig. 4.1), but not for the proportion of flowers setting fruit. Overall, 96% of the 174 experimental flowers produced fruit, including all intact flowers (i.e., capable of autogamy) that also received supplemental outcross pollen. The proportion of flowers setting fruit did not differ overall among treatments ($\chi^2 = 5.57$, d.f. = 3, $P = 0.134$),

colours ($\chi^2 = 2.45$, d.f. = 1, $P = 0.118$), populations within colour ($\chi^2 = 2.12$, d.f. = 3, $P = 0.346$), or between specific combinations of treatment and colour (interaction: $\chi^2 = 4.13$, d.f. = 3, $P = 0.248$) or treatment and population(colour) (interaction: $\chi^2 = 4.04$, d.f. = 9, $P = 0.672$).

Overall, 81% of ovules developed into seeds; however, seed set differed significantly between red- and pink-flowered populations ($\chi^2 = 5.40$, 1 d.f., $P = 0.020$) and among treatments in two of the four populations (treatment x population(colour); $\chi^2 = 28.31$, 6 d.f., $P < 0.001$: Fig. 4.1). Plants from red-flowered populations produced more seeds per ovule that those from pink-flowered populations (Fig. 4.1), but proportional seed set did not differ overall between populations within colours ($\chi^2 = 1.10$, 2 d.f., $P = 0.577$). Overall, seed set differed among pollination treatments ($\chi^2 = 183.2$, 1 d.f., $P < 0.001$), being lower for flowers that could only self-pollinate autonomously than for those subject to hand-pollination. However, this variation occurred only for plants from the two pink-flowered populations (Fig. 4.1). In the GG population, plants in the unmanipulated treatment set fewer seeds than intact flowers that also received cross-pollen. As a consequence, this population had the smallest IAF (Table 4.1), indicating greater pollinator dependence than for the other populations. In the MH population, autonomously selfing flowers set fewer seeds than cross-pollinated emasculated flowers. Consequently, this population had the largest ISI (Table 4.1), indicating greater benefit from cross-pollination.

Mechanism and timing of autonomous self-pollination

As at least 50% of ovules in flowers that could only self-pollinate autonomously in the previous experiment developed into seeds (Fig. 4.1), I examined when and how self-pollination occurred. Anthers and stigma lobes were most separated in young flowers and herkogamy declined significantly as flowers aged (Treatment $\chi^2 = 151.22$, 2 d.f., $P < 0.001$, all pairwise $P < 0.001$: Fig. 4.2 a). Consequently, although some autonomous self-pollination was evident for flowers emasculated 24 and 48 h after anthesis, stigmas of flowers with intact anthers during their last day received ten-fold more pollen ($\chi^2 = 183.15$, 2 d.f., $P < 0.001$: Fig. 4.2 b). Seed production showed a similar pattern. Fruits of flowers emasculated on the second or third day of flowering developed almost three times fewer seeds than flowers that were not emasculated (Treatment $\chi^2 = 63.53$, 2 d.f. = 2, $P < 0.001$: Fig. 4.2 c).

Inbreeding depression

Hesperantha coccinea exhibits limited inbreeding depression. Performance of selfed and outcrossed progeny did not differ statistically for 16 of 18 comparisons for three populations, including for seed production, germination rate, maximum leaf height of seedlings and mature plants, and number of inflorescences (Fig. 4.3, Table 4.2). The exception involved production of significantly more flowers by outcrossed progeny than by selfed progeny in one red- and one-pink-flowered population (Fig. 4.3e). Overall, performance differed among populations for four of the six measured life stages, with consistent treatment effects in all populations (all Treatment*population interactions $P > 0.7$; Table 4.2). Based on these results, the estimated cumulative inbreeding depression differed among populations from -0.01 to 0.25 (Table 4.2).

Mating system

Populations of both ecotypes exhibited mixed mating, but the multilocus estimate of outcrossing (± SE) for GG ($t_m = 0.695 \pm 0.075$) was double that at EL ($t_m = 0.373 \pm 0.084$). The diversity of male mates within seed families was also greater at GG than at EL (correlation of paternity ± SE, $r_p = 0.55 \pm 0.17$ at EL and 0.18 ± 0.07 at GG). Consistent with greater self-fertilisation at EL, its coefficient of inbreeding was higher (F_{IS} ± SE across all loci = 0.52 ± 0.06) than at GG (0.18 ± 0.05). The correlation of selfing among loci (r_s) indicated greater biparental inbreeding at EL: realised selfing at GG was exclusively uniparental ($r_s \pm SE = 1.00 \pm 0.05$), whereas biparental inbreeding accounted for approximately a quarter of realised selfing at EL ($r_s = 0.73 \pm 0.06$).

Discussion

Both ecotypes of *Hesperantha coccinea* exhibit characteristics consistent with "Best-of-Both-Worlds" reproduction. In addition to producing large, showy flowers that attract insect pollinators (Chapter 2), populations of both ecotypes can self-pollinate autonomously (Fig. 4.2), self-pollination readily fertilizes ovules (Fig. 4.1), and selfed zygotes develop into reproductive adults with similar success to outcrossed zygotes (Fig 4.3). Importantly, most autonomous self-pollination occurs late during anthesis of individual flowers (Fig. 4.2b), allowing pollen export and outcross pollen priority access to ovules when pollinators visit

frequently. When instead pollinators visit infrequently, delayed autogamy assures ovule fertilisation with little seed discounting. Coupled with limited genetic load in populations, as indicated by the weak inbreeding depression, these characteristics should allow both ecotypes of *H. coccinea* to realize high female success, regardless of mate and pollinator abundance, while also being able to competitively sire seeds on other plants in suitable pollination and mating environments. Evidence for BOBW reproduction in both ecotypes indicates that the pollinator shift in this species has occurred in spite of autofertility and was potentially mediated by the same selective forces that maintain pollinator specialisation in BOBW reproduction.

Self-compatibility and autofertility

Autogamy can provide reproductive assurance only if it is coupled with self-compatibility, which is clearly a feature of *H. coccinea* (Fig. 4.1). Self-compatibility combined with autofertility occurs widely within the genus (Goldblatt 1984, Goldblatt et al. 2004) and family: Among the Iridaceae, showy species of *Sparaxis*, *Moraea*, *Babiana* and *Lapeirousia* with highly specialised pollination systems are also self-compatible and autogamous (Anderson et al. 2005, Goldblatt and Manning 2006). Of the dozen (out of c. 80) *Hesperantha* species for which self-compatibility has been quantified two species are self-incompatible; four are partially self-compatible, showing reduced fecundity following self-pollination; one species with a highly specialised beetle-pollination system is partially autofertile; and four species are completely autofertile, experiencing no fecundity reduction when isolated from pollinators (Goldblatt 1984, Goldblatt et al. 2004, van Kleunen et al. 2008). Despite evidence that floral adaptations in the genus reflect shifts in pollination system (Goldblatt et al. 2004), both self-compatibility and autofertility are common, which suggests that BOBW reproduction is more widespread in the genus.

Delayed self-pollination

In the first instance, “Best-of-Both-Worlds” reproduction requires floral mechanisms that enable delayed self-pollination. Approximately 80% of showy selfing species exhibit such mechanisms (Fenster and Martén-Rodríguez 2007). Reduced herkogamy during the lives of individual flowers, such as that observed in *H. coccinea* (Fig. 4.2c), is among the most widespread mechanisms enabling delayed selfing in pollinator-specialised species (Goodwillie and Weber 2018). Recurvature of the style branches bringing stigmatic surfaces

into contact with anthers in older flowers, such as observed for *H. coccinea*, has also been observed in other southern African irid taxa but consequences for autofertility have seldom been quantified in detail (Goldblatt and Manning 2006). Interestingly, although flowers of *H. coccinea* close overnight on consecutive nights (Goldblatt et al. 2004), reorientation of reproductive parts appears to ensure that most autonomous self-pollination only occurs at the end of floral life (Fig. 4.2). Given the high fruit and seed set observed in two red- and two pink-flowered *H. coccinea* populations (Fig. 4.1), capacity for delayed autogamy seems to be a general feature of this species. However, this capacity may vary quantitatively among populations, as bagged flowers of plants from the two pink-flowered populations set relatively fewer seeds than those subjected to hand-pollination (Fig. 4.1). This contrast seems to be associated with pollination success, rather than differential survival of selfed zygotes, as no inbreeding depression was evident during seed development (Fig. 4.3a). This difference suggests heterogeneity among *H. coccinea* populations in the extent or timing of reduced herkogamy.

Realised outcrossing rates confirm that delayed self-pollination likely contributes to mixed mating in both ecotypes of *H. coccinea*. However, marker-based estimates of mating system do not distinguish autogamous selfing from geitonogamous pollinator-mediated selfing, which does not confer the same adaptive advantages (Lloyd 1992). Floral manipulations to quantify the reproductive assurance benefit of selfing (Lloyd and Schoen 1992) would be useful to confirm whether selfing in *H. coccinea* indeed functions to provide reproductive assurance in unpredictable pollination environments (Kalisz and Vogler 2003), and to investigate whether mating in *H. coccinea* conforms to predictions of Johnson 2009 with regard to functional relationships between fitness components. Although variation in mating among populations is not uncommon (Whitehead et al. 2018) the almost twofold difference in selfing rates between the sampled populations of *H. coccinea* pollinated by butterflies ($s_m = 0.627 \pm 0.084$ at EL) and long proboscid flies ($s_m = 0.305 \pm 0.075$ at GG) respectively, suggests potential for differences in mating characteristics associated with pollinator behaviour and ecology (cf. Kulbaba and Worley 2012, Kulbaba and Worley 2013, Krauss et al. 2017, Rhodes et al. 2017). Investigation of mating system characteristics from additional populations of each ecotype would be useful in this regard.

Inbreeding depression

Self-fertilisation after autonomous self-pollination provides reproductive assurance only to the extent that the resulting zygotes develop into reproductive adults. This is the case for the studied *H. coccinea* populations, which exhibited low inbreeding depression overall and during all measured life stages, except for flower production in two populations (Fig. 4.3, Table 4.2). Limited inbreeding depression during early life stages and comparatively elevated inbreeding depression during later stages occurs commonly among species that predominately self-fertilise (Husband and Schemske 1996). That performance was specifically higher for flower production by outcrossed progeny compared to selfed progeny is noteworthy. Importantly, the 15% greater average flower production by outcrossed progeny has broader implications for offspring reproduction, including greater ovule and pollen production, and increased opportunity to interact with more pollinators and mates. Thus, the consequences for the relative fitness of outcrossed progeny may be proportionally greater than suggested by the measured difference in flower number.

Like most similar studies, my experiment likely underestimated inbreeding depression, which depends somewhat on environmental conditions (Cheptou and Donohue 2011). In particular, the performance differences between selfed and outcrossed progeny observed under greenhouse conditions may be less than would occur in more variable and less benign field environments (Armbruster and Reed 2005). Nevertheless, it seems highly unlikely that selfed progeny in natural *H. coccinea* populations experience strong (i.e., > 0.5) inbreeding depression. For example, in their review of studies that compared inbreeding depression in benign and stressful environments, Armbruster and Reed (2005) reported significant differences for only 48% of cases. Assessment of post-dispersal inbreeding depression using the difference in inbreeding coefficients of adult plants and seeds for large samples of seed families from natural populations (Ritland 1990, Koelling et al. 2012) would be informative in this regard.

Low inbreeding depression, such as observed for *H. coccinea*, is not unprecedented compared to other mixed-mating species (Winn et al. 2011), but its potential significance for BOBW mixed mating has been hitherto underappreciated (Herlihy and Eckert 2002, Kalisz et al. 2012, Goodwillie and Weber 2018). One explanation for adaptative maintenance of traits associated with specialised pollination in BOBW species is that high inbreeding depression in BOBW limits the benefits of selfing (Goodwillie and Weber 2018). Empirical quantifications

of inbreeding depression in BOBW species, although limited, do not support this expectation. Although a few studies have found evidence that costs of inbreeding limit reproductive benefits of autofertility (e.g. Herlihy and Eckert 2002, Vaughton et al. 2008), a recent survey of inbreeding depression in species known to exhibit delayed selfing found low inbreeding depression (ID < 0.3) for all nine species with estimates available from early life stages only, and for more than half of the fourteen species for which cumulative inbreeding depression has been quantified (Goodwillie and Weber 2018). Low inbreeding depression can be consistent with BOBW reproduction if fitness components other than female outcrossing are considered, specifically, if specialisation reflects selection acting through male reproductive success (Fenster and Martén-Rodríguez 2007). In showy selfers characterised by high autofertility and weak inbreeding depression, both quantity and quality components of pollen limitation (Aizen and Harder 2007) and thus, variation in female success (c.f. Bateman 1948) are reduced. Under these conditions, pollinator-mediated pollen export that occurs prior to autonomous self-pollination can confer a fitness advantage through siring success. Importantly, whereas maternal success is limited by ovules and resources of an individual plant, pollen export enables access to ovules of all coflowering individuals in a population and is limited by pollinator activity (Bell 1985). Thus, the potential magnitude of the fitness advantage through siring success, is much greater than through female fecundity, and could play an important role in maintenance of pollinator adaptations in showy selfers in which pollen limitation is reduced by autofertility and low inbreeding depression (also see Stanton et al. 1986, Paterno et al. 2020). Low inbreeding depression in this study and other putative BOBW species (e.g. Kalisz and Vogler 2003, reviewed in Goodwillie and Weber 2018) suggests that inbreeding depression can and does vary widely in BOBW species (see also Johnston et al. 2009) and that siring success may represent a key but unrecognised advantage of pollinator specialisation in "Best-of-Both-Worlds" reproduction (see also Fenster and Martén-Rodríguez 2007). Insight into the roles of inbreeding depression and male fitness in the maintenance of pollinator specialisation in BOBW species will benefit from further studies which quantify selection through male fitness and female fitness components (Winn et al. 2011), as well as studies which explore the extent to which autofertility alleviates pollen limitation of female success in BOBW taxa.

Pollinator shift in an autofertile species

This evidence for BOBW reproduction in both ecotypes of *H. coccinea* suggests that the pollinator shift in this species has occurred in spite of autofertility and supports the idea that reproductive assurance offered by delayed selfing may represent an additional route contributing to the frequency of pollinator shifts in flowering plants (van der Niet and Johnson 2012, Wessinger and Kelly 2018). The characteristics of BOBW found to accompany the pollinator shift in this study in *H. coccinea* are consistent with the predictions of theoretical models (Wessinger and Kelly): firstly, and critically, self-pollination is delayed (prior and competing selfing do confer the same advantages for a pollinator shift), and secondly, autofertility and the relative fitness of selfed offspring is high, both of which broaden potential for pollinator shifts in self-compatible species (Wessinger and Kelly 2018). Pollinator shifts enabled by delayed selfing are further predicted to be disproportionately associated with shifts to rare but highly efficient pollinators, and to involve fixation of recessive advantageous alleles associated with loss of function mutations (Wessinger and Kelly 2018). Data is inadequate to assess either of the latter predictions for the pollinator shift in *H. coccinea*, however, since *H. coccinea* is the only butterfly-pollinated *Hesperantha* species, it seems likely that the shift in this species is from fly pollination to butterfly pollination. Quantification of pollen deposition from single visits to unpollinated flowers suggests both pollinators are similarly effective in terms of pollen receipt (Chapter 2), but the consequences of the two pollination systems for pollen export efficiency are unknown. Interestingly, the shift in *H. coccinea* appears to involve evolution of red flowers, as is also the case in the evolution of hummingbird pollinated flowers involving loss of function mutations in several other genera (Wessinger and Kelly 2018 and references therein), but the nature of the pigments and genetic basis of evolution of red flower colour in *H. coccinea* is unknown.

Since the pollination ecotypes of *H. coccinea* are conspecific (Goldblatt and Manning 1996b), the evidence presented here that both ecotypes exhibit characteristic specialised pollination (Chapter 2) and delayed autofertility (this chapter) suggests that BOBW reproduction was likely a relatively stable ancestral trait, which predates the pollinator shift. This evidence for BOBW at an ancestral node suggests that BOBW reproduction can be maintained over at least the time frame required for floral adaptation to a novel pollinator, and therefore contributes evidence in favour of the long-term stability of BOBW strategies (Goodwillie et al. 2005).

Tables

Table 4.1 Indices of self-incompatibility (ISI) and autofertility (IAF) for four populations of *Hesperantha coccinea*.

Population	Flower colour	ISI	IAF
Bushman's Nek (BN)	Red	0.13	0.89
Elliot (EL)	Red	-0.08	0.83
Mahai (MH)	Pink	0.15*	0.72
Golden Gate (GG)	Pink	-0.06	0.55†

* Associated with a significant difference in proportional seed set between intact flowers not subject to hand-pollination and those that received supplemental outcross pollen.

† Associated with a significant difference in proportional seed set between intact flowers not subject to hand-pollination and emasculated flowers hand-pollinated with outcross pollen.

Table 4.2 Estimated inbreeding depression at six life stages and cumulative inbreeding depression, and details of the associated statistical analyses for three populations (Bushman's Nek, BN; Elliot, EL; Kamberg, KM). of *H. coccinea*. Asterisks indicate that the estimated inbreeding depression is associated with significant performance differences between selfed and outcrossed progeny within a population for the associated life stage.

	Inbreeding Depression			Treatment			Population			Treatment*population		
Life Stage	BN	EL	KM	χ^2	df	p	χ^2	df	p	χ^2	df	p
Seeds developed	0.01	-0.02	0.09	0.09	1	n.s.	1.24	2	n.s.	0.60	2	n.s.
Germination rate	-0.09	0.02	-0.14	0.35	1	n.s.	8.02	2	< 0.05	0.30	2	n.s.
Seedling height	-0.13	-0.07	-0.05	0.99	1	n.s.	7.02	2	< 0.05	0.11	2	n.s.
Mature plant height	0.03	-0.03	-0.03	0.12	1	n.s.	38.56	2	< 0.001	0.66	2	n.s.
Number of flowers	0.08	0.14*	0.15*	9.39	1	< 0.01	50.15	2	< 0.001	0.71	2	n.s.
Number of inflorescences	0.07	0.07	0.21	0.57	1	n.s.	13.55	2	= 0.001	0.23	2	n.s.
Cumulative	**-0.01**	**0.12**	**0.25**									

Figures

Figure 4.1

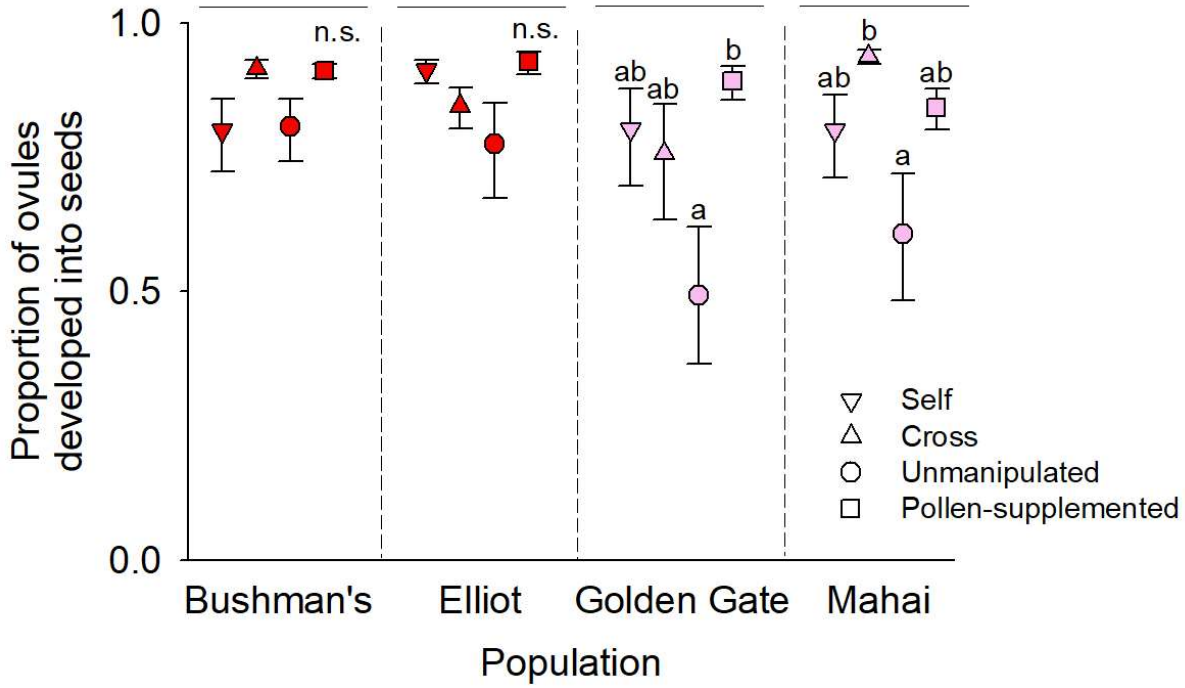

Fig. 4.1 Mean ± SE proportions of *H. coccinea* ovules that developed seeds for emasculated flowers pollinated with self-pollen (downward facing triangles), emasculated flowers pollinated with cross-pollen (upward triangles), unmanipulated flowers (circles) and flowers hand-pollinated with supplemental cross-pollen (squares). Plants were maintained in a pollinator-free greenhouse. Red and pink symbols indicate flower colour in each population. Contrasting letters indicate significant differences for pairwise contrasts between treatments within a population, based on sequential Šidák correction.

Figure 4.2

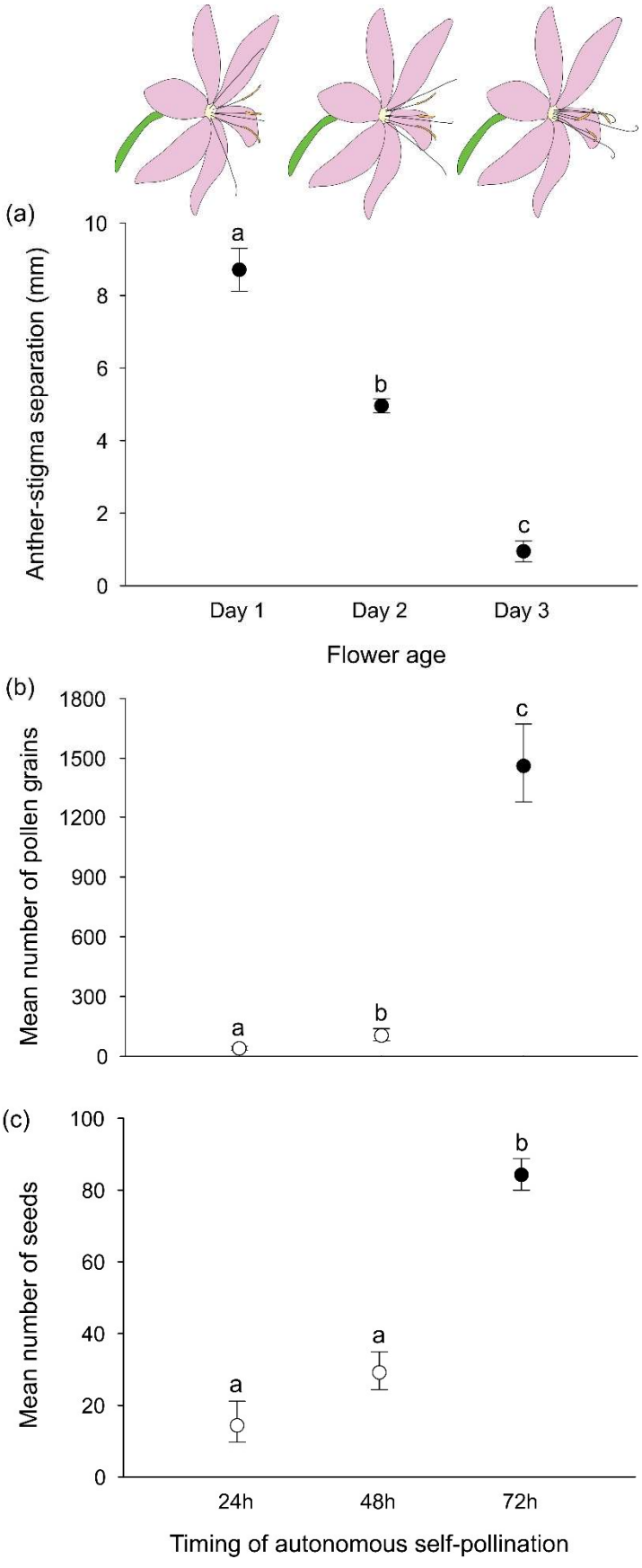
(a)
Anther-stigma separation (mm)
10
8
6
4
2
0
a
b
c
Day 1
Day 2
Day 3
Flower age
(b)
Mean number of pollen grains
1800
1500
1200
900
600
300
0
a
b
c
(c)
Mean number of seeds
100
80
60
40
20
0
a
a
b
24h
48h
72h
Timing of autonomous self-pollination

Fig. 4.2 Mechanism and timing of autonomous self-pollination by *H. coccinea* flowers. Changes in mean ±SE (a) stigma-anther separation, (b) pollen grains on stigmas and (c) number of developed seeds. In panel (a) images illustrate changes in stigma orientation over floral life. In panels (b) and (c) open symbols indicate flowers that were emasculated such that self-pollination was only possible until 24 or 48 hours after anthesis, filled symbols indicate flowers that were not emasculated, for which autonomous self-pollination was possible until the last morning of anthesis. Different letters indicate significant differences for pairwise contrasts between treatments, based on sequential Šidák correction.

Figure 4.3

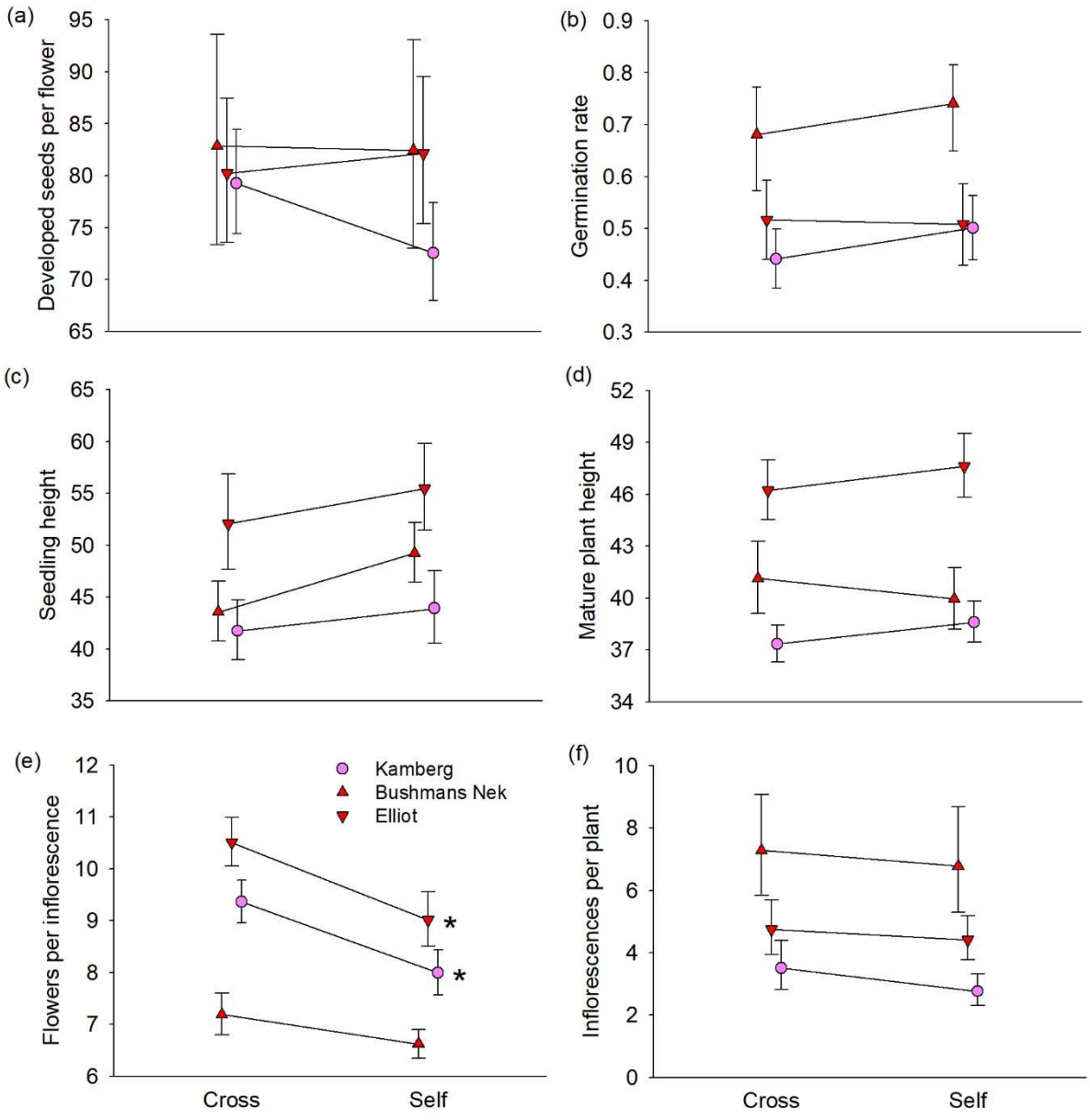

Fig. 4.3 Comparison of mean ±SE performance of selfed and outcrossed *H. coccinea* seeds and progeny from three populations, including (a) number of developed seeds, (b) germination rate, (c) seedling height at 3 months, (d) mature plant leaf height, (e) flower number, and (f) number of inflorescences per seed family. Red and pink symbols indicate flower colour in each population. Asterisks indicate significant differences between treatments within a population. See Table 4.2 for statistical details.

Appendix

Table A4.1 Numbers of treated *Hesperantha coccinea* plants from four populations and of flowers in each treatment of a greenhouse experiment that used hand pollination to assess self-compatibility and autofertility.

				Pollination treatment			
			Number	Emasculated		Intact	
Population	Location		of plants	Self	Cross	Unmanipulated	+ Cross-pollen
Bushman's Nek (BN)	-29.843S	29.209E	21	19	16	18	19
Elliot (EL)	-31.313S	27.867E	11	6	5	10	11
Mahai (MH)	-28.696S	28.906E	11	9	6	8	9
Golden Gate (GG)	-28.507S	28.620E	11	10	10	9	9

Table A4.2 Sample sizes for the quantification of inbreeding depression during successive life stages for *Hesperantha coccinea* plants from three populations, including the numbers of hand-pollinated flowers (developed seeds) and of maternal families (germination, number of inflorescences) and offspring (all other life stages).

		Life stage					
Population	Treatment	Developed seeds	Germination rate	Seedling height	Mature height	Number of inflorescences	Flowers per inflorescence
Bushman's Nek	Cross	7	7	7	7	7	21
	Self	7	9	9	9	9	28
Elliot	Cross	14	14	14	13	8	13
	Self	14	14	12	12	10	17
Kamberg	Cross	26	25	25	22	8	21
	Self	25	21	20	19	9	27

Table A4.3 Details of nine microsatellite loci used to estimate outcrossing rates for two populations of *Hesperantha coccinea* at Elliot (EL) and Golden Gate (GG), including; loci combined into each multiplex reaction, range in base-pairs, numbers of alleles (numbers in parentheses indicate population-specific alleles), observed (H_O) and expected (H_E) heterozygosity, coefficient of inbreeding (F_{IS}) and number of progeny genotyped. Bold numbers indicate H_O/H_E ratios associated with significant deviation from Hardy-Weinberg equilibrium.

		Repeat		Range	N alleles			H_O/ H_E		F_{IS}		N progeny	
Locus	Primer sequence (5' – 3')	motif	5' Dye	(bp)	Total	EL	GG	EL	GG	EL	GG	EL	GG
Multiplex 1													
Hc3	F: TTTGGTTCTTGCTCTCTCCA	CA	NED	191- 205	3	2	1	0.05/		0.640	-0.275	130	155
	R: CATGACCAAGAAGGCACAAA					(2)	(1)	0.13					
Hc5	F: GGTACGTTGCATAATAAATCACCA	TC	HEX	186 – 240	15	13	3	0.55/	0.52/	0.338	-0.275	129	155
	R: TGACAATGCATGGGTCAAT					(10)	(1)	0.83	0.41				
Hc6	F: GGTACGTTGCATAATAAATCACCA	CA	HEX	145 – 171	9	4	2	**0.12/**	0.29/	0.775	0.377	111	154
	R: TGACAATGCATGGGTCAAT					(3)	(2)	**0.55**	0.47				
Hc8	F: FAM-GTTTCGGTTTTGGAACGATG	CT	FAM	146 – 206	12	10	1	0.45/	**0.00/**	0.465	1.000	129	156
	R: GAGGCTGGAATCTCGTCAAA					(6)	(1)	0.84	**0.14**				
Hc12	F: GCAATTCATATTTCATCCAAGC	GT	PET	187 – 195	3	3	1	0.40/		0.300		130	153
	R: TTTCCTCCAGCCAACAGAAC					(1)		0.57					

Table A4.3 continued

Locus	Primer sequence (5' – 3')	Repeat motif	5' Dye	Range (bp)	N alleles Total	N alleles EL	N alleles GG	H_O/ H_E EL	H_O/ H_E GG	F_{IS} EL	F_{IS} GG	N progeny EL	N progeny GG
Multiplex 2													
Hc11	F: TCAACCAGACTGAAAGGAAGG	TC	FAM	221 – 227	4	4	4	**0.25/**	0.47/	0.511	0.209	132	153
	R: TTCAATGATTTGCCAAGGAG							**0.51**	0.59				
Hc16	F: AACAGCTGAAACTGCAGTCAAAC	GT	HEX	210 – 232	4	3	1	0.30/		0.394		131	154
	R: CCCTATTATGGCCTATTCACTTG					(3)	(1)	0.49					
Hc19	F: GCTAACACGACTTCCTACTGGTC	AG	PET	148 – 172	8	6	2	**0.44/**	0.25/	0.345	0.467	125	157
	R: TCCATACGGTGAAATTCAGAAAC					(6)	(2)	**0.67**	0.46				
Hc22	F: CAATAATCTCTCCTCCTTTGCTG	CT	NED	157 – 183	10	8	2	**0.27/**	**0.05/**	0.616	0.634	127	156
	R: AGTCCTTCCAGGATTAAGATTCG					(8)	(2)	**0.72**	**0.16**				

Appendix A4.4 Molecular methods and selection of study populations for estimation of mating system in *Hesperantha coccinea*

Populations of each ecotype suitable to use to estimate mating system parameters were selected as follows. Seventeen maternal plants at Elliot and 20 from Elands Heights, Kamberg and Golden Gate were screened with eleven loci previously developed for *H. coccinea* (Wolff et al. 2009). Nine loci were combined with minimal optimisation into two PCR multiplexes, with forward primers labelled with M13 florescent dyes (Applied Biosystems, Foster City, USA). Locus combinations and dyes for the multiplex reactions are provided in Table A4.3. Each 15-µl multiplex reaction contained 6 µl dH_2O, approximately 2 ng of DNA and 1pmol of each primer in 9 µl of KAPA2G Multiplex Mix (SigmaAldrich). The amplification procedure was 95° (3 min) followed by 30 cycles of 95° (15 s), 60° (30 s) and 72° (30 s) with a final extension at 72° for 10 min. Fragment analysis was conducted with a 50 capillary Applied Biosystems ABI3130xl Genetic Analyser with Hi-Di Formanide and LIZ500 size standard (Applied Biosystems, Foster City, USA). Allele variation and range were determined by manual scoring using GeneMarker V2.2.1 (SoftGenetics, LLC^{TM}). All samples, except eight from KM, amplified successfully. For the successfully amplified samples, nine loci were polymorphic at EL (mean ± SE, 4.9 ± 0.9 alleles per locus), eight at EH (3.9 ± 0.7), six at GG (2.0 ± 0.4) and two at KM (0.3 ± 0.2). Due to financial and time constraints, quantification of outcrossing rates was limited to one population of each ecotype. Based on ease of amplification, number of informative loci and allelic richness EL and GG were considered suitable representative populations.

GenAlEx (Peakall and Smouse 2012) was used to calculate observed and expected heterozygosity and assess deviation from Hardy Weinberg equilibrium (HWE). Several loci exhibited heterozygote deficiency and significant ($p < 0.05$) deviation from HWE, but with one exception these differed between populations (Table A4.3). Heterozygote deficiency may reflect biological realities, and is seldom considered justification to discard loci (Jarne and David 2008). At GG and EL maternal coefficients of inbreeding (F_{IS} ± SE: GG, 0.24 ± 0.14; EL, 0.35 ± 0.08) are consistent with a history of inbreeding, and the loci were retained.

CHAPTER 5: THE CONTRIBUTIONS OF POLLINATORS AND AUTONOMOUS SELF-FERTILISATION TO REPRODUCTION ACROSS THE RANGE OF A SHOWY SOUTH AFRICAN IRIS

Abstract

Plants subject to unpredictable pollination can benefit from 'Best-of-Both-Worlds reproduction' (BOBW), possessing traits that both promote outcrossing and the capacity for delayed self-fertilisation. Despite accumulating empirical support for the benefits of BOBW reproduction, the actual contributions of pollinators and delayed selfing to seed production have seldom been quantified across species distribution ranges. Thus, the extent to which variation in pollinator service contributes to stable BOBW reproduction remains to be confirmed. I investigated multiple populations of two pollination ecotypes of the showy autofertile irid *Hesperantha coccinea* to characterise temporal and spatial variation of pollinator failure, reproductive assurance from delayed selfing (RA), and pollen limitation. Specifically, I considered the relations of these outcomes to population elevation, population peripherality and floral traits indicative of investment in pollinator attraction or associated with autonomous self-fertilisation. For both pollination ecotypes, autonomous self-fertilisation ameliorated the effect of pollinator failure on reproductive success, but pollen limitation was still evident in the pink-flowered ecotype. Overall, approximately 75% of seeds resulted from pollinator-mediated pollination and 25% from autonomous self-fertilisation. A significant contribution of autonomous self-pollination was evident in 11 of the 15 populations sampled, but for nine of the 11, significant RA was detected in only one or some of the sampled seasons. The contribution of self-fertilisation to fecundity varied among populations from being undetectable to accounting for 96% of developed seeds and varied within populations by an average of 28% year to year. Pollinator failure did not vary consistently geographically or with floral display, flower size or herkogamy. Autonomous self-fertilisation is a consistent component of evolutionarily stable BOBW reproduction in *H. coccinea*.

Key words: Reproductive assurance, pollen limitation, pollinator failure, Best-of-both-worlds reproduction, pollination ecotypes, *Hesperantha coccinea*, Iridaceae.

Introduction

The diversity of plant mating systems, which range from complete outcrossing to exclusive selfing is commonly associated with variation in floral phenotypes (Stebbins 1970, Barrett 2002). In particular, for animal-pollinated species the extent to which reproduction depends on pollinators versus autonomous self-fertilisation varies with investment in floral traits that function in pollinator interaction (Goodwillie et al. 2010) or mediate autonomous self-pollination (Ornduff 1969). Large displays of colourful flowers with separated sex organs typically characterise self-incompatible, obligately outcrossing species that rely completely on pollinators to reproduce (Willmer 2011). In contrast, self-compatible species that rely predominantly on self-fertilisation are characterised by a "selfing syndrome" of fewer, smaller, inconspicuous flowers with little anther-stigma separation and reduced investment in colour, scent and rewards (Sicard and Lenhard 2011). Correlations between outcrossing rates and flower size and number support an association of reliance on selfing with decreased allocation to floral display traits (Goodwillie et al. 2010). The role of pollinator failure in generating these patterns has been demonstrated experimentally, with increased ability to self-fertilise and changes in associated floral traits evolving in response to pollinator exclusion in as few as five generations (Bodbyl Roels and Kelly 2011)

"Showy selfers" present an exception to the rule that investment in floral displays reflects reliance on pollinators for reproduction. Such species combine showy flowers with the ability to reproduce by autonomous self-fertilisation that usually occurs after outcrossing and thus allows intermediate outcrossing rates (mixed mating). This apparent paradox has been hypothesised to represent a "Best-of-Both-Worlds" (BOBW) strategy that allows reproductive flexibility in unpredictable pollination environments: adaptations for pollinator attraction maximise pollinator-mediated outcrossing, whereas delayed self-fertilisation provides reproductive assurance when pollinator service is limited (Darwin 1862, Fenster and Martén-Rodríguez 2007). Mechanisms of delayed self-pollination have been documented for more than 65 taxa with showy flowers (Goodwillie and Weber 2018). However, many putative cases of BOBW reproduction also exhibit characteristics inconsistent with this strategy. In particular, many species with delayed autonomous self-fertilisation also show reduced allocation to floral attractiveness and evolution of selfing-syndrome traits compared to predominantly outcrossing sister taxa (reviewed in Goodwillie 2018, e.g. Robertson and Lloyd 1991, Jesson and Barrett 2002, Mazer et al. 2007). As interspecific differences in floral variation and mating systems can reflect influences of factors other than plant-pollinator

interaction, studies of intra-specific variation could be useful for understanding differences in floral variation and mating (Eckert et al. 2006, also see Whitehead et al. 2018), especially for putative BOBW species (Goodwillie et al. 2010, Goodwillie and Weber 2018). Studies of species capable of delayed selfing that compare floral traits among populations or intraspecific forms with contrasting mating patterns often find that plants in highly outcrossing populations have larger flowers and greater herkogamy than those in more selfing populations (e.g. Elle and Carney 2003, Button et al. 2012, Dart et al. 2012). Further, one comparison of populations with contrasting pollinator activity and importance of autonomous selfing revealed that greater reliance on autonomous selfing weakens pollinator-mediated selection for large flowers and attractive traits (Teixido and Aizen 2019). Thus, evidence consistent with maintenance of traits for pollinator attraction has been demonstrated for few, if any, putative BOBW species. Furthermore, whether "showy selfers" exhibit an optimal BOWB strategy or are undergoing transition to predominant selfing remains a subject of debate (Igic and Kohn 2006, Goodwillie and Weber 2018).

Joint quantification of geographic and temporal variation in pollinator service and reproductive assurance would help distinguish patterns consistent with shifts to selfing from those consistent with BOBW reproduction. Geographic differences in pollinator availability, especially reduced pollinator service at range edges or high elevation, are thought to be a major driver of shifts to predominant selfing (Stebbins 1970, also see Moeller 2006, Perez et al. 2013). In contrast, with BOBW reproduction selection should maintain traits for both pollinator attraction and autonomous self-pollination to contend equally with unpredictable low and high pollinator availability. Thus, a key prediction of BOBW reproduction is that the relative contributions of pollinator-mediated pollination and autofertility to reproduction vary with pollination service but not with floral traits that govern either process. Few studies include the replication of emasculation experiments among populations and years required to test this expectation (reviewed in Goodwillie and Weber 2018). Further, among the few studies that have investigated relations between reliance on selfing for reproduction and investment in floral traits in BOBW systems, several use outcrossing rates to assess mating outcomes (Goodwillie and Weber 2018). This metric does not distinguish autonomous selfing from pollinator-mediated selfing within- or among-flowers, which require the same floral characteristics as outcrossing (Lloyd 1987, Eckert 2000). Thus, factors such as pollinator abundance, population size and density, and marginal or peripheral location of populations within a species' range could influence pollinator-mediated selfing similarly to autonomous selfing. Consequently, direct quantification of the reproductive assurance benefit of selfing,

requires comparison of seed production by emasculated and intact flowers (Schoen and Lloyd 1992) to distinguish the autonomous and pollinator contributions to reproductive success (e.g. Kalisz et al. 2004, Ruan et al. 2011) and their relevance to the evolution of floral traits and plant reproductive systems.

Ecotypic differences of floral traits in *Hesperantha coccinea*, an irid species, seem to represent contrasting adaptations for near-exclusive pollination by either long-proboscid flies of the genus *Prosoeca* or large Nymphalid butterflies (Chapter 2 and 3). Although these traits are consistent with adaptation for pollinator-mediated cross-pollination, both ecotypes also exhibit self-compatibility and capacity for reproduction independent of pollinators via delayed self-pollination (Chapter 4). This combination of specialised animal pollination and capacity for reproduction independent of pollinators suggests BOBW reproduction by *H. coccinea*. However, the contributions of pollinators and autonomous self-fertilisation to reproductive success have not been assessed under natural field conditions. Therefore, to investigate whether reproduction in *H. coccinea* is consistent with BOBW mating across the species range, I quantified pollinator failure, pollen limitation and reproductive assurance in 15 populations during two to four flowering seasons. Specifically, I asked: 1. Does pollinator failure vary among populations and annually within populations, as required for BOBW reproduction to be beneficial? 2. To what extent does autonomous self-fertilisation offset pollinator failure and provide reproductive assurance that reduces pollen limitation of seed production for both pollination ecotypes? 3. Do pollinator failure and the importance of autonomous self-fertilisation for reproduction increase towards range margins or high elevation? 4. Does the importance of pollinators and autonomous self-fertilisation for reproduction correlate among populations with floral traits that mediate pollinator attraction (flower size, display size) and autonomous reproduction (herkogamy)?

Materials and Methods

Study species and sites

Hesperantha coccinea (Iridaceae) occurs along water courses along the Drakensberg and Great Escarpments in southern Africa (Goldblatt 1993). Flowers in most populations are either red or pink, but a few populations include mixtures or intermediates of these flower colours (Goldblatt 1993, Goldblatt and Manning 1996b). Controlled pollination experiments

have shown that the showy flowers of both ecotypes are self-compatible and autofertile, with high seed set independent of pollinators (Chapter 4). Fifteen populations, including six of the red-flowered ecotype, seven of the pink-flowered ecotype and two mixed populations, were selected to encompass the floral variation and geographic range of *H. coccinea* (see Chapter 2). Locations of the study sites are shown in Fig. 5.1 and co-ordinates are provided in Table A5.1 in the supplementary material.

Quantification of pollinator failure, autonomous self-fertilisation and pollen limitation

With BOBW mating, cross-pollination should vary negatively with local pollinator visitation, whereas autonomous selfing should vary positively. I assessed this variation among populations of both ecotypes throughout the species range based on comparisons of seed production by flowers subject to one of three conditions (Fig. 5.2). "Natural pollination" and "emasculated" treatments were applied to paired flowers on individual plants in 2 to 15 populations during one to four flowering seasons between 2009 and 2012 (Table A5.1). Natural pollination flowers were unmanipulated and represent seed production resulting from the prevailing incidence of pollinator-mediated and self-pollination. Anthers of flowers assigned to the emasculation treatment were removed with forceps from mature buds just prior to flower opening, preventing autonomous self-pollination. Thus, seed production by emasculated flowers is exclusively attributable to cross-pollination resulting from pollinator activity. An additional "pollen supplementation" treatment to assess maximal fecundity was applied in three populations during 2011 and in all 15 populations during 2012 (Table A5.1). These flowers received cross-pollen from one to three donor plants at least 5 m from the treated plant. Supplemental pollen was applied by brushing anthers against receptive stigmas with enlarged stigmatic papillae. All treatment flowers were exposed to natural pollination for the duration of flowering. Manipulative treatments were applied during up to three days to allow rotation of treatment positions in an inflorescence. Experimental flowers were marked by attaching a coloured wire around the base of the floral tube.

Approximately six weeks after treatments were applied, fruit set (presence / absence) was noted, fruits were harvested and all the seeds were counted. The number of seeds per fruit, including zeros for flowers that failed to produce fruit, was used in subsequent analyses. Experiments involved a total of 1484 flowers on 567 inflorescences (mean per population per year, 20.25 flowers per treatment, range eight to 35 inflorescences). Details of sampling in each population and season are provided in Table A5.1.

Contrasts of seed production by the three treatments additionally measure three relevant aspects of female reproductive success within a population.

Pollinator failure is the extent to which female reproductive success was limited by pollinator activity in a population, as represented by the difference in mean seed production between supplementally pollinated flowers ($\bar{S}_s$) and emasculated flowers ($\bar{S}_e$) (Fig. 5.2). To facilitate comparison among populations, this difference was standardised by dividing by mean seed production by supplemented flowers,

$$PF = \frac{\bar{S}_s - \bar{S}_e}{\bar{S}_s} = 1 - \frac{\bar{S}_e}{\bar{S}_s}$$

(Kalisz and Vogler 2003, Brys and Jacquemyn 2011).

Reproductive assurance is the contribution of autonomous self-fertilisation to seed production, as represented by the difference in mean seed-production between naturally pollinated flowers ($\bar{S}_n$) and emasculated flowers. Following common practice (Lloyd and Schoen 1992, Kalisz and Vogler 2003), this difference was standardized by dividing by mean natural seed production,

$$RA = \frac{\bar{S}_n - \bar{S}_e}{\bar{S}_n} = 1 - \frac{\bar{S}_e}{\bar{S}_n}.$$

Pollen limitation is the extent to which pollen receipt limits seed production, which was calculated as the mean fecundity difference between supplementally pollinated and naturally pollinated flowers. The relative index of pollen limitation was standardised by dividing by mean seed production by supplemented flowers,

$$PL = \frac{\bar{S}_s - \bar{S}_n}{\bar{S}_s} = 1 - \frac{\bar{S}_n}{\bar{S}_s}$$

(Lloyd and Schoen 1992). Note that $PF \neq PL + RA$, because the denominator of *RA* differs from that of *PF* and *PL*.

Geographic patterns of pollinator service and autonomous self-fertilisation

To determine whether higher pollinator failure and greater reliance on autonomous self-fertilisation vary with proximity to the range periphery or population elevation, geographic variables of peripherality and elevation were included as covariates in analyses of seed set for the full 2012 dataset of the emasculation, pollen supplementation and control treatments in 15 populations. *Hesperantha coccinea* occurs between 800 and 2200 m.a.s.l. along

approximately 1600 km of the Drakensberg escarpment. Because the species distribution is roughly linear north to south, and characterised by minimal longitudinal variation (See Chapter 2), I used the latitude of each population and the northernmost or southernmost known population, whichever was closer, to quantify proximity to a range limit ("peripherality" sensu Eckert et al. 2008). Northernmost and southernmost populations were identified by mapping all georeferenced herbarium specimens from herbaria in South Africa (PRE, NBG, BOL, NU), the United Kingdom (K) and the Netherlands (AMD, L, U, WAG). The study populations spanned 1000 m elevation and 900 km of latitude (Fig. 5.1).

Associations between floral traits and importance of autonomous self-fertilisation

I also considered the associations of population mean PF, RA and PL to average reproductive traits. Flower size and number (inflorescence size) both affect pollinator attraction (Conner and Rush 1996, Ohashi and Yahara 2001, Ishii and Harder 2006) and herkogamy mediates autonomous self-pollination (Ornduff 1969, Sicard and Lenhard 2011). Size traits were measured using digital callipers to 0.01-mm precision (Fig. 5.3). Inflorescence size was recorded as the sum of the number of buds, open and wilted flowers on an inflorescence for 529 inflorescences (each from a different plant) in 15 populations (mean ± SE = 35.3 ± 3.9 plants per population, range = 12 to 67). Flower size was calculated as the product of tepal length (from the mouth of the floral tube to the petal tip) and width (at the widest point: Fig. 5.3) for one flower from each of 442 plants (mean ± SE 29.5 ± 2.7 plants per population, range 15 to 54). Flowers of *H. coccinea* have three anthers and three stigma branches, which separate at the mouth of the floral tube. The receptive regions (stigmatic surfaces) are located on the apical half of each stigma branch. Herkogamy was measured as the shortest distance between any anther and a stigmatic region on a stigma branch (Fig. 5.3) on one randomly selected flower per plant on 344 flowers in 15 populations (mean = 22.9 ± 2.4 plants per population, range 6 to 43). Because different plants were sampled for floral measurements and pollination treatments, all treated flowers were assigned their population trait mean for statistical analyses (see below). Sampling details, population means and results of ANOVAs for each trait are provided in Appendix Table A5.2 in the supplementary material.

Tests of assumptions concerning floral emasculation

Elevated success of intact flowers versus emasculated is commonly interpreted as measuring the contribution of autonomous self-fertilisation and hence reproductive assurance to seed

production (Lloyd, 1992); however, emasculation can cause other unintended effects, leading to overestimation of RA (Schoen and Lloyd 1992). Such effects include reduced pollinator visitation, especially if presence of pollen is important for pollinators, and flower damage that reduces floral lifespan or potential fecundity (Dart and Eckert 2013b). I assessed these possible side-effects by comparing seed set following hand pollination, floral longevity and pollinator attraction by intact and emasculated flowers.

Experiments to determine whether floral damage during emasculation affects fecundity were conducted using plants from four populations (BN, EL, GG, MH) maintained in a pollinator-free environment in the Botanical Gardens of the University of KwaZulu-Natal's Pietermaritzburg campus. One to three flowers on each of 49 plants were assigned to an emasculation or unmanipulated treatment. Receptive stigmas of flowers were hand pollinated with pollen from at least one donor plant from a maternal seed family that differed from that of the recipient. After approximately four weeks, fruit set was recorded and the number of developed seeds was counted for all fruits.

The effects of emasculation on floral longevity and pollinator attraction were examined in a natural population at Golden Gate (GG) in February 2013. To assess longevity, treatments were assigned randomly to two unopened flowers on each of 30 inflorescences. Anthers of the flowers assigned to the emasculated treatment were removed from mature buds just prior to flower opening. Whether experimental flowers had wilted was examined twice daily, during early morning, c. 09:00 h, and afternoon just prior to closing at c. 16:00 h (Goldblatt et al. 2004). In young flowers, tepals of open fresh flowers are displayed perpendicular to the floral tube. In contrast, the tepals of wilting flowers were too close together to allow access to a pollinator (<2 cm).

To determine whether emasculation affects pollinator attraction, an inflorescence with two open intact flowers and another with two open emasculated flowers were placed in florist's spike vases 60 cm apart at the end of a 1-m pole and presented to pollinators as they visited flowers in the population (Chapter 2, Thomson 1988). The positions of intact and emasculated inflorescences were alternated between presentations. Inflorescences were offered to individuals of the three most frequently recorded insect visitors to *H. coccinea* (Chapter 2), *Aeropetes tulbaghia* (n = 24), *Prosoeca ganglbaueri* (n= 15), and *Macroglossum trochilus* (n = 23 foraging bouts). Each time a foraging insect was offered the treated inflorescences, the treatment of the first flower visited and the number of visits to flowers of each treatment were recorded.

Data analysis

Analyses to assess the effect of emasculation on fecundity, floral longevity and pollinator attraction involved generalised linear models, as implemented in SPSS 27 {IBM \Corp., 2020 #734}. Generalised estimating equations with an exchangeable correlation matrix were used to account for correlations among flowers within inflorescences (Liang and Zeger 1986). Counts of seeds were modelled with the negative binomial distribution and ln link function. Flower longevity was modelled with the Gaussian distribution. Insect choices (first flower visited) in relation to the number of approaches, and the number of visits to emasculated flowers in relation to the total number of flowers visited during each foraging bout were modelled with the binomial distribution and logit link function.

The effects of the pollination treatments and other influences on seed production per flower were assessed with generalised linear mixed models (Stroup 2013), as implemented in the glmmTMB package (version 1.0.2.1; Brooks et al. 2017) of R (version 4.0.3; R Core Team 2020). AIC comparisons of full models involving Poisson, negative binomial or Tweedie distributions, all with ln link functions, identified the Tweedie distribution as most appropriate, and it was used in all analyses reported here. In addition to pollination treatment, all analyses included plant within population as a random factor to account for sampling of multiple flowers per individual. Most analyses involved plant samples from multiple years, so year and its interaction with treatment were included as fixed effects. Similarly, most analyses also considered plants from multiple populations. Population differences were considered as fixed effects for analyses involving few populations or for which population estimates were the subject of interest, or as random effects when the overall variance among populations associated with geography or floral traits was the subject of the analysis. Population ecotype and its interaction with treatment were also initially included in analyses that included both red- and pink-flowered populations. Ecotype could be explicitly excluded from final models, based on AIC, but it was still implicitly represented in the specific differences among populations.

As the pollination experiments were conducted in different populations and years and did not all include supplemental pollination (Table A5.1), I conducted separate analyses to assess year and population effects on seed production from different perspectives. For example, the four annual samples of the natural and emasculated treatments in the KM population provided the most thorough assessment of inter-year variation in reproductive assurance. In contrast, the 2012 samples for all three treatments from all 15 populations

provided the most thorough assessment of among-population variation in pollinator failure, reproductive assurance and pollen limitation. When presenting the results for a specific analysis, I identify the relevant combination of treatments, years and populations and the perspective that it addresses.

The 15-population sample for 2012 was used in several analyses to assess the effects of pollination treatments and population characteristics. The analysis to compare seed production between the red- and pink-flowered ecotypes considered population within ecotype as a random factor. As the sample included three types of populations (red, pink and mixed), I used a priori orthogonal contrasts to specifically compare the red- and pink-flowered populations (Kirk 1995). To quantify whether overall differences among populations in elevation, proximity to a range limit, mean display size, flower size or herkogamy (each ln-transformed) influenced treatment effects, population was also included as a random factor. In contrast, an analysis to characterize the specific treatment effects within each population considered population as a fixed effect. Ecotype could not be included in this analysis, owing to convergence problems during model fitting.

Effects and random terms (plant identity, population, and colour) were excluded from models if they were not involved in any significant interactions ($\alpha = 0.05$) and their elimination resulted in an improvement in model AIC of 2 or more. Results from only the best-fit model are reported. Significant interactions were explored further with Tukey adjustment for unplanned contrasts and Dunn-Šidák for planned multiple comparisons (Kirk 1995).

Contrasts of mean seed production between the different pollination treatments can be used to estimate relative pollinator failure (PF), reproductive assurance (RA) and pollen limitation (PL). As the glmm analyses involved ln link functions, back-transformation of the mean difference for two treatments represents the ratio of the untransformed means. Therefore,

$$PF = 1 - e^{\ln\bar{S}_e - \ln\bar{S}_s}, \quad 1a$$

$$RA = 1 - e^{\ln\bar{S}_e - \ln\bar{S}_n}, \quad 1b$$

$$PL = 1 - e^{\ln\bar{}_n - \ln\bar{S}_s}. \quad 1c$$

I used the *R* emmeans package (version 1.5.2-1; Lenth 2020) to estimate the marginal ln mean differences and their 95% confidence intervals. The statistical significance of an index estimate was assessed based on whether its associated confidence interval excluded zero. The approach summarised in equation 1 was also used to characterise the regression relations of

the indices to continuous covariates. In this case, the treatment means in equation 1 were replaced by the glmm estimates for individual covariate values (e.g., $\ln\hat{S}_n = a_n + b_n X$).

Results

Tests of emasculation assumptions

Floral longevity, fecundity and attractiveness did not differ statistically between emasculated and unmanipulated flowers (Table A5.3). On average, emasculated flowers lasted 4.48 days (lower SE [LSE] 4.21, upper SE [USE] 4.75) and intact flowers wilted after 4.52 days (LSE 4.29, USE 4.83). Fruits from emasculated flowers contained an average of 87.9 seeds (LSE 83.8, USE 92.3) compared to 88.9 seeds for intact flowers (LSE 86.2, USE 91.7). For all insect species, approximately half of both the first flowers chosen and all flowers visited were emasculated (first choice, mean 50%, 95% CI = 37 – 62%; all visits, mean 52%, CI = 41 – 62%). These results did not differ among appreciably among visitor species (Table A5.3, Fig. A5.1). Based on these results, differences in seed production by emasculated flowers from that of either naturally or supplementally pollinated flowers likely reflect the elimination of autonomous self-pollination by emasculation.

Variation among 15 populations

Overall, seed production during 2012 by *H. coccinea* differed among the three pollination treatments. As a consequence, insufficient or poor-quality pollinator visitation reduced seed production 41% below the maximum capacity evident for flowers that received supplemental pollination (overall PF = 0.41, 95% CI = 0.35 – 0.46). Even though autonomous self-fertilisation provided appreciable reproductive assurance (RA = 0.27, 0.19 – 0.34), overall seed production by naturally pollinated flowers remained 19% below maximum capacity (PL = 0.19, 0.11 – 0.26).

For both pollination ecotypes, pollination treatment affected the reproductive success of flowers and its effect was consistent for both ecotypes (Fig. 5.4a, Table 5.1). Reproductive success of pollen supplemented flowers did not differ between ecotypes, indicating similar reproductive potential (t_1 = -0.69, P = 0.492; Fig. 5.4), but seed production for both emasculated flowers and for unmanipulated flowers was higher for the red-flowered ecotype than for the pink-flowered ecotype (emasculated, t_1 = -2.02, P = 0.04, unmanipulated t_1 = -2.65, P = 0.01; Fig. 5.4a). In populations of the pink-flowered ecotype, pollinator activity was

sufficient to fertilise 57% of ovules (PF = 0.43, autonomous self-fertilisation accounted for approximately a quarter of seed set (RA = 0.23), and 26% of ovules remained unfertilised due to pollen limitation (PL = 0.26). In populations of the red-flowered ecotype, pollinator activity was sufficient to result in fertilisation of 68% of ovules (PF = 0.32), approximately 28% of developed seeds resulted from autonomous self-fertilisation (RA = 0.28), and a non-significant proportion of ovules remained unfertilised (PL = 0.06). In both red- and pink-flowered pollination ecotypes, significant pollinator failure and reproductive assurance were evident; however, significant pollen limitation was observed only in the pink-flowered ecotype (Fig. 5.4b).

Overall seed set varied among the 15 populations sampled during 2012 (Table 5.1, Fig. 5.5a). However, this variation largely involved low seed production by three populations, one red-flowered (KR) and two pink-flowered (DL, PJ), including in response to pollen supplementation. Otherwise, flowers on pollen-supplemented plants produced about 90 seeds. In contrast, seed production by naturally pollinated and emasculated flowers varied more extensively among populations (Fig. 5.5a).

Treatment differences among populations resulted in considerable heterogeneity in pollinator failure, reproductive assurance and pollen limitation. In five populations, including populations of both ecotypes (red, BN and KR; pink, KM and PJ) and a mixed population (NK), pollinator failure was alleviated by reproductive assurance (Fig. 5.5b: confidence intervals exclude 0). In contrast, three pink-flowered populations (DH, MM, DL), experienced intense pollinator failure and pollen limitation, but did not benefit from reproductive assurance (Fig. 5.5b). Results from seven of the populations were ambiguous with regards to BOBW reproduction. In a pink-flowered (GG) and a mixed population (GC), pollinator failure limited reproductive success, but neither PL nor RA was statistically significant. Plants in the remaining five populations, including four red-flowered (HG, EH, EL, WK) and one pink-flowered (MH), apparently receive sufficient pollinator visitation to maximize seed set during 2012, as their confidence intervals for PF, RA and PL all included zero (Fig. 5.5b).

Inter-year variation

Analysis of seed set for three populations (GG, KM, NK) in which all three treatments were applied during 2011 and 2012, detected effects of treatment, population and year (Table 5.1). Overall, flowers in the mixed population (NK) set somewhat fewer seeds than those in the

two pink-flowered populations (Fig. 5.6a), but population differences were not evident among years or treatments (population interactions, Table 5.1). In contrast, treatment effects differed between years (Table 5.1). During 2011, PF was not evident in any of the three focal populations, whereas significant PF limited reproductive success in all three of the focal populations in 2012 (Fig. 5.6b). Autonomous self-fertilisation alleviated PF to the extent that PL was not detected in either year (Fig. 5.6b).

Four analyses exclusively considered the effect of emasculation on seed production compared to that of naturally pollinated plants during multiple flowering seasons (Table 5.2). In an analysis of nine populations during 2011 and 2012, the effects of treatment varied among combinations populations and years (Table 5.2). Across all populations during 2011, approximately 50% of seeds resulted from pollinator mediated pollen transfer and 50% from autonomous self-fertilisation (RA = 0.49, 0.37 – 0.58). In contrast, during 2012, 75% of seeds resulted from pollinator activity and 25% from autonomous self-pollination (RA = 0.25, 0.15 – 0.33; Fig. 5.7a). Pollinator-mediated seed set differed between years for four of the nine populations, whereas for intact flowers between-year differences in seed set were observed in only two populations, owing to the mediating effect of autonomous self-fertilisation (Table A5.4). The mean within-population difference in RA between years was 0.28 (95% confidence interval 0.09 – 0.47, n=9). Reproductive assurance differed most between years in population MM, where autonomous self-fertilisation contributed 93% of seeds during 2012 (RA = 0.93), but was non-significant during 2011 (RA = 0.02, Fig. 5.7b). RA exceeded 0 in five of the nine populations during 2011 (EH, NK, DH, MH, MM) and four in 2012 (BN, NK, KM, GG; Fig. 5.7b). RA differed between years in only two (DH and MM) of the nine populations. For the remaining seven populations, RA was statistically significant during only one year (Fig. 5.7b).

For three populations, RA was also quantified during 2009 and/or 2010 (Fig. 5.8). At BN and KM, seed production did not vary among years, overall or for individual treatments (Table 5.2, Fig. 5.8a). However, naturally pollinated flowers consistently produced more seeds than emasculated flowers, resulting in similar reproductive assurance in both populations (BN, RA = 0.25, 0.12 – 0.36; KM, RA = 0.26, 0.13 – 0.37: Fig. 5.8b). In contrast, at GG seed production did not differ between treatments or years (Table 5.2, Fig. 5.7a), so autonomous self-fertilisation did not contribute significantly (GG RA = 0.10, -0.06 – 0.24). Despite the absence of treatment by year interactions (Table 5.7), significant RA was observed consistently during all years that KM and BN were sampled (Fig. 5.8b). At GG,

where autonomous self-fertilisation did contribute overall (Table 5.2), significant RA was detected during one of the three years (Fig. 5.8b).

Effects of site and population characteristics

Analyses of the 15-population data for 2012 that also considered site characteristics found no relation of seed production to population proximity to range limits, but a possible association with population elevation (Table A5.3). Although seed production did not vary overall with population elevation, the interaction of elevation and treatment was statistically significant (Table A5.3). Specifically, seed set of emasculated flowers decreased with increasing elevation (partial regression coefficient $\beta_{emasculated} = 1.09$, 95% CI = 0.23 – 1.94), whereas that of intact and pollen supplemented flowers did not vary with elevation ($\beta_{intact} = 0.34$, -0.46 – 1.16; $\beta_{supplemented} = 0.22$, -0.57 – 1.02). Consequently, reproductive assurance increased statistically with elevation ($\beta = 1.09$, 0.23 – 1.95, Fig. 5.9a), whereas pollen limitation ($\beta =$ 0.34, -0.47 – 1.16) and pollinator failure ($\beta = 0.23$, -0.57 – 1.02) varied independently of elevation (Fig 5.9b). However, these results seem particularly influenced by the lowest site (KR). Specifically, the elevation x treatment interaction was not statistically significant when KR was excluded from the analysis (Table A5.5).

Population differences in seed production and treatment responses also seem unrelated to variation in mean floral characteristics. None of the 15-population analyses that included population mean flower number per inflorescence, flower size or herkogamy and their interactions with treatment detected statistically significant effects (Table A5.6).

Discussion

Populations of both pollination ecotypes of *H. coccinea* are characterised by Best-of-Both-worlds reproduction whereby a combination of autonomous self-fertilisation and pollinator-mediated reproduction optimises reproductive success under variable pollination conditions. In particular, as indicated by variation in seed set of emasculated flowers, autonomous self-fertilisation increased fecundity in the face of substantial variation in pollinator service among populations and flowering seasons (Table 5.1 and 5.2, Fig. 5.4 – 5.8). Reproductive assurance alleviated limited seed set imposed by pollinator failure for populations of both the pink- and red-flowered ecotypes, but it eliminated pollen limitation only for the red ecotype.

The relative importance of pollinators and autonomous self-fertilisation for fecundity also did not vary among populations in association with proximity to range edges or floral traits involved in pollinator attraction or autonomous self-pollination (Table A5.5 and A5.6, Fig. 5.9), consistent with autonomous self-fertilisation as a flexible component of optimal Best-of-Both-Worlds reproduction.

Best-of-Both-Worlds reproduction

BOBW reproduction involves reproduction by means of pollinator-mediated pollen transfer to the extent that pollinator service allows, complemented by autonomous self-fertilisation when pollinator service does not maximise seed set. Consistent with these expectations, pollinator-mediated pollination was responsible for c. 73% of seed set by *H. coccinea* and the remainder resulted from autonomous self-pollination (Fig. 5.4). Despite the significant contribution of autonomous self-fertilisation in alleviating pollinator failure, seed set remained pollen limited overall, being 20% less than maximal capacity. Both pollination ecotypes experienced significant overall pollinator limitation of reproductive success and realised reproductive assurance benefits of autonomous self-fertilisation. However, pollen limitation was generally avoided in red-flowered populations, owing to almost complete reproductive assurance, whereas assurance was only partial for the pink-flowered ecotype.

Population estimates of PF, RA, and PL suggest underlying differences in pollinator service between ecotypes. During 2012, pollinator-mediated pollination alone was sufficient to eliminate pollen limitation of seed production in four of six red-flowered populations, but only two of seven pink-flowered populations (Fig. 5.5b). During all years, significant RA was evident in 11 of the 15 populations; three of six red-flowered populations and six of seven pink-flowered populations experienced significant RA during at least one year. Among all populations during all years sampled, the relative importance of pollinator and autonomous self-fertilisation ranged from 96% reliance on self-fertilisation to no discernible benefit of autonomous self-fertilisation in populations in which pollinator-mediated pollination maximized seed production within ovule or resource limitation (Fig. 5.5 – 5.8). In addition, the reproductive assurance benefit of autonomous self-fertilisation typically differed by approximately 30% between years within a population (mean 0.28, 95% confidence limits 0.09 – 0.47, n = 9).

Within-population, inter-year differences in seed-set by emasculated flowers are consistent with the prediction that BOBW is associated with temporal variation in pollinator

service. Such variation in the importance of pollinators and autonomous self-fertilisation for reproduction may underlie the lack of clear influences of population geography and floral traits (Table A5.5 and A5.6). Seed set of emasculated flowers and the reproductive assurance benefit of autonomous self-fertilisation may increase at lower elevations; however, this trend was not accompanied by a parallel trend in pollinator failure and the significance of this trend depended on a single population, so it is difficult to interpret. Overall, the results suggest that both ecotypes are characterised by BOBW reproduction, relying primarily on pollinators for pollination, but having the backup of delayed self-pollination. The absence of geographic structure or associations with changes in floral traits suggest that BOBW is a stable strategy in *H. coccinea*.

BOBW reproduction allows plants to maximize reproduction in variable pollination environments (Becerra and Lloyd 1992) subject to pollinator failure during some flowering seasons or parts seasons but not during others (Kalisz and Vogler 2003, Ruan et al. 2008). . In several populations, absence of pollinator failure indicated high levels of pollinator service (Fig. 5.5). Complete absence of pollinator failure was more frequent in populations of the red-flowered ecotype (HG, EH, EL WK) than for those of the pink-flowered ecotype, for which such adequate pollinator service was evident for only one population (MH), and for two of the three populations (EH and MH, but not EL) for which no pollinator was detected in 2012, reproductive assurance did contribute to fecundity in 2011 (Fig. 5.7), confirming that autonomous self-fertilisation does contribute to fecundity despite high pollinator service in some years. Such unpredictable pollination service is essential for BOBW reproduction to be beneficial.

Single-season estimates cannot demonstrate the inter-year variation in PF and autonomous self-fertilisation that are essential requirement for BOBW reproduction. In this study, most experiments that quantified RA for multiple seasons in a population detected RA in one season but not in another (seven out of nine populations: Fig. 5.5, 5.7 and 5.8). Similarly, although only five of the 15 populations investigated in 2012 experienced significant PF, it was evident in one season but not in the other for all populations that were studied during a second season (Fig. 5.5 and 5.6). This inter-year heterogeneity suggests that RA might also be a feature of reproduction in the (four) populations for which it was not observed, even though it did not occur during the few years when sampling occurred. In at least one of these populations (EL), previous work has shown equivalent fecundity in the absence of pollinators to that in response to supplemental cross-pollination, indicating very high (ability to set seed in absence of pollinators, sensu Lloyd and Schoen 1992, Eckert et al.

2010, Chapter 4). Other studies that have used larger samples also detected RA during some time periods (early or late in the flowering season) or in some populations but not others (Kalisz and Vogler 2003, Ruan et al. 2011). These results support for the expectation that the combination of showy flowers and delayed selfing evolves to maximize reproduction in variable pollination environments (Kalisz and Vogler 2003, Fenster and Martén-Rodríguez 2007, Goodwillie and Weber 2018).

Geographic patterns of pollinator service and autonomous self-fertilisation

Effective pollinator service is generally expected to decline and selfing be more prevalent in peripheral populations (Baker 1955) or those at high elevation (Totland 2001). Many demonstrated clines across latitude or elevation support these expectations (e.g. Jain 1976, Wyatt 1988, Moeller 2006, Moeller et al. 2012). In contrast, neither pollinator-mediated seed set nor autonomous self-fertilisation varied among 15 *H. coccinea* populations distributed across more than 400 km from the range centre to the periphery (Fig. 5.1., Table A5.5). Indeed, flowers in two of the most peripheral populations near the species' southern range limit (HG and EH) produced similar numbers of seeds after pollen supplementation or emasculation (Fig. 5.1 and 5.5). Similarly, the trend for higher seed set by emasculated flowers, which are pollinated exclusively by pollinators, in higher populations is counter to the expectation of more pollinator failure in the more extreme conditions at high elevation (Warren 1988). The range of *H. coccinea* reaches only c. 2200 m.a.s.l., and thus may not include the alpine environments associated with depauperate pollinator communities (Arroyo et al. 1985, Perez et al. 2013, Koch et al. 2020). Higher *H. coccinea* populations also tend to be pollinated by Diptera (Chapter 2), which are generally more dominant pollinators at higher elevations (Lefebvre et al. 2018). Alternatively, the detected relation may not reflect a true trend in pollinator service, as the influential low elevation site also differs from other sites in that the original grassland habitat has been transformed to commercial forest plantation, which may have affected pollinator service. More data from additional low elevation sites are required to determine whether decreased pollinator availability characterizes lower elevation sites.

The limited geographic patterning of pollinator failure, reproductive assurance and pollen limitation for *H. coccinea* are consistent with BOBW. Shifts to increased reliance on self-fertilisation and the evolution of selfing syndrome phenotypes typically reflect a response to consistent pollinator failure at range edges or reduced pollinator availability

following colonization of a novel environment (references). In other species that reproduce predominantly by outcrossing, highly autofertile, selfing races occur where an original pollinator is absent (Jain 1976, Wyatt 1988, Moeller 2006) or in association with reduced pollinator service due to other factors such as competition (e.g. Fishman and Wyatt 1999, Buide et al. 2015). These selfing races are characterized by reduced flower size, anther-stigma separation and investment in pollinator attraction: floral traits which characterize the selfing syndrome (Sicard and Lenard 2011). In contrast, even *H. coccinea* populations closest the range periphery experience appreciable pollinator service (Fig. 5.1, Fig. 5.7). Furthermore, inter-year variation in pollinator service (Fig. 5.6 – 5.8) suggests variable selection for increased self-fertilisation.

Floral trait variation and autonomous self-fertilisation

Neither the pollinator-mediated nor the autonomous contributions to seed production varied among populations in association with mean flower number and size or herkogamy (Table A5.6). This result contrasts with the findings of many previous studies (e.g. Webb and Lloyd 1986, Herlihy and Eckert 2007, Brys and Jacquemyn 2011, Schouppe et al. 2017, Torang et al. 2017, reviewed in Koski et al. 2019) and with the broader trend that higher selfing rates are associated with reduced allocation to investment in pollinator attraction (e.g. Tang and Huang 2007, Button et al. 2012, reviewed in Goodwillie et al. 2010). Both flower size and anther-stigma separation have high potential to evolve fast in response to selection (Opedal 2019) and have been shown to change in a few generations in response to changes in the pollination environment (Bodbyl Roels and Kelly 2011, Gervasi and Schiestl 2017). Thus, the absence of associations between reliance on self-fertilisation and herkogamy, or reciprocally, between the importance of pollinators for reproduction with flower size or number, strongly suggest that the phenotype of *H. coccinea* is an optimal Best-of-Both-Worlds phenotype. The absence of selfing rate-selfing trait associations in *H. coccinea* suggest that traits that mediate autonomous selfing and pollinator attraction are not associated with strong trade-offs, such that both can be maintained together. Consideration of the mechanism of self-pollination suggests that increased self-pollination effectiveness might not impose costs to pollinator attraction: self-pollination in *H. coccinea* is mediated by reduced anther-stigma separation towards the end of the four days of floral anthesis (Chapter 4), with little consequence for outcrossing.

Many studies that reported associations between selfing rates and floral traits considered molecular estimates of overall selfing rates (Goodwillie et al. 2010), which conflate pollinator-mediated selfing and autonomous self-fertilisation rates. Only the autonomous component of selfing results in reduced selection for pollinator attraction and increased selection for traits that mediate effective self-pollination (Lloyd 1979). The prevalence of associations between selfing rate and floral traits revealed by this study, which explicitly quantified autonomous self-fertilisation, is perhaps more surprising. The extent to which RA represents the realised autonomous selfing rate in *H. coccinea* is unknown. Comparisons of selfing rates estimated with SSRs and the results of emasculation experiments from two populations suggest that geitonogamous and pollinator-mediated intrafloral self-pollination contribute substantially to realised selfing. For one red-flowered population (Elliot EL) and one pink-flowered population (Golden Gate GG), the estimated selfing rates were 0.67 and 0.37, respectively (Chapter 4), whereas reproductive assurance during the same seasons was 0.18 and 0.11 (this chapter). Capacity for autofertility and timing of autonomous self-pollination are key factors that could affect whether RA estimates in *H. coccinea* accurately represent the contributions of autonomous self-pollination to reproduction. Both vary among populations in other species (Spigler 2018, Ruane et al. 2020, also se Yang et al. 2018) and preliminary results suggest this may also be true in *H. coccinea*. Among four populations, the autofertility metric AF varied from 0.57 to 0.9 (Chapter 4). Experiments in a single population suggest that in *H. coccinea* most autonomous self-pollination occurs towards the end of floral life (Chapter 4). Further work dissecting the components of realised selfing (Eckert 2000, Brunet and Sweet 2006) is required to determine whether the absence of association between reliance of autonomous selfing and floral traits found in this study holds when more accurate measures for autonomous selfing rates are used.

The observation that PL persists in some populations despite high autofertility, and that this may differ between ecotypes has implications for how and why the pollinator shift may have occurred in *H. coccinea*, as well as for diversification in mixed mating species in general. The greater frequency of pollinator failure in populations of the pink-flowered ecotype (Fig. 5.4 and 5.5) suggests that the shift to pollination by butterflies may have been favoured because it alleviated pollinator limitation, especially as populations dispersed beyond the predominantly high-elevation range of the *Prosoeca* pollinators of pink-flowered ecotype (Chapter 2). The persistence of pollen limitation is particularly interesting, because it suggests that both maintenance of adaptive traits in BOBW systems in general and the

particular case of a shift to a novel pollinator in *H coccinea* may have occurred through both male and female functions, even though autofertility alleviates pollen limitation (Darwin 1862, Knight et al. 2005, e.g. Briscoe Runquist and Moeller 2013). Although male success provides an additional alternative pathway for pollinator adaption in BOBW species (Fenster and Martén-Rodríguez 2007, also see Bell 1985, Ashman and Morgan 2004), this study shows that even in autofertile BOWB species, PL and thus potentially selection through both gender components may maintain showy floral traits. The overall contribution of pollinators to 75% of seed set found in this study is consistent with an important role of pollinators for reproductive success, despite high autofertility (Chapter 4, this chapter). A caveat is that PL and PF in this study may be overestimated because of the methods used: Estimates are based on single flowers rather than whole plant treatments and may therefore underestimate PL (Ashman et al. 2004), and do not take into account potential for resource reallocation (Briscoe Runquist and Moeller 2013) or pollen quality effects (Aizen and Harder 2007). However, fruit and seed set are uniformly high in *H. coccinea* (in this study, >90% of naturally pollinated flowers set fruit and ovules develop into seeds), which minimises potential for reallocation. Furthermore, pollen origin does not affect seed set (Chapter 4), so it seems likely that pollen and pollinator limitation have played a role in pollinator adaption in *H. coccinea* and that the mechanism may also function more generally in maintenance of floral adaptations for pollinator-mediated reproduction in autofertile species (also see Wessinger and Kelly 2018).

Conclusion

This study demonstrates that both ecotypes of *H. coccinea* engage in BOBW reproduction, and that pollinators and autonomous selfing both contribute to seed production across the species range. Based on this study, *H. coccinea* resembles many showy species for which autofertility provides reproductive assurance (Goodwillie and Weber 2018). In addition, this study reveals novel implications. Firstly, for many showy species characterised by delayed self-fertilisation, variation in floral traits among populations reflects the importance of selfing for reproduction (reviewed in Goodwillie and Weber 2018), often along geographic clines of pollinator availability (Runions and Geber 2000, Busch 2005 but see Koski 2017). Reduced flower size in association with greater reliance on selfing is also found in species that engage in stable mixed mating (e.g. Dart et al. 2012). In contrast, in *H. coccinea*, a BOBW species,

flower size and number and herkogamy vary independently among populations with variation in reliance on autonomous self-fertilisation. The lack of association between floral traits or geography and reproductive assurance in *H. coccinea* suggests optimality of the "showy selfer" phenotype, potentially mediated by an absence of strong trade-offs between selfing and outcrossing traits in this species. Secondly, known examples of autofertile sister taxa typically differ in selfing ability and selfing-syndrome traits (e.g. Brys and Jacquemyn 2011, Briscoe Runquist et al. 2017, Schouppe et al. 2017), whereas in *H. coccinea*, the presence of BOWB in both pollination ecotypes and in mixed populations point parsimoniously toward an ancestor with a mixed mating system.

Figures

Figure 5.1

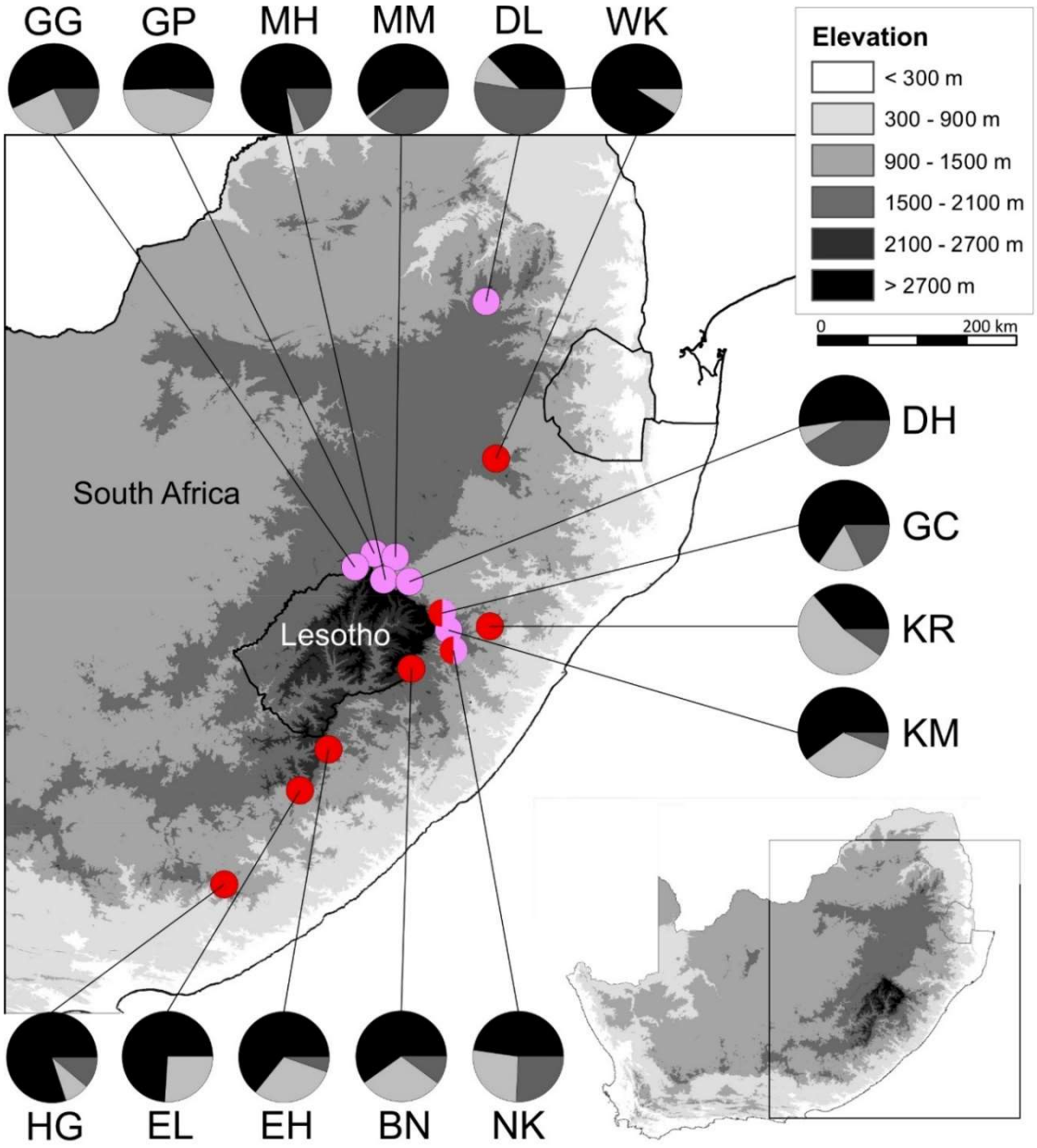

Fig. 5.1 Geographic variation in the proportion of seeds resulting from pollinator mediated and autonomous pollination, and the proportion of undeveloped seeds as a result of pollen-limitation in 15 populations of *H. coccinea*. Pie charts show the mean proportion of ovules that develop into seeds as a result of pollinator activity (black), autonomous self-fertilisation (light grey) and that are unfertilised due to pollen limitation (dark grey) in each population. Symbols indicate locations of study sites and flower colour at each site. Rectangle in the inset shows the location of the study area within South Africa.

Figure 5.2

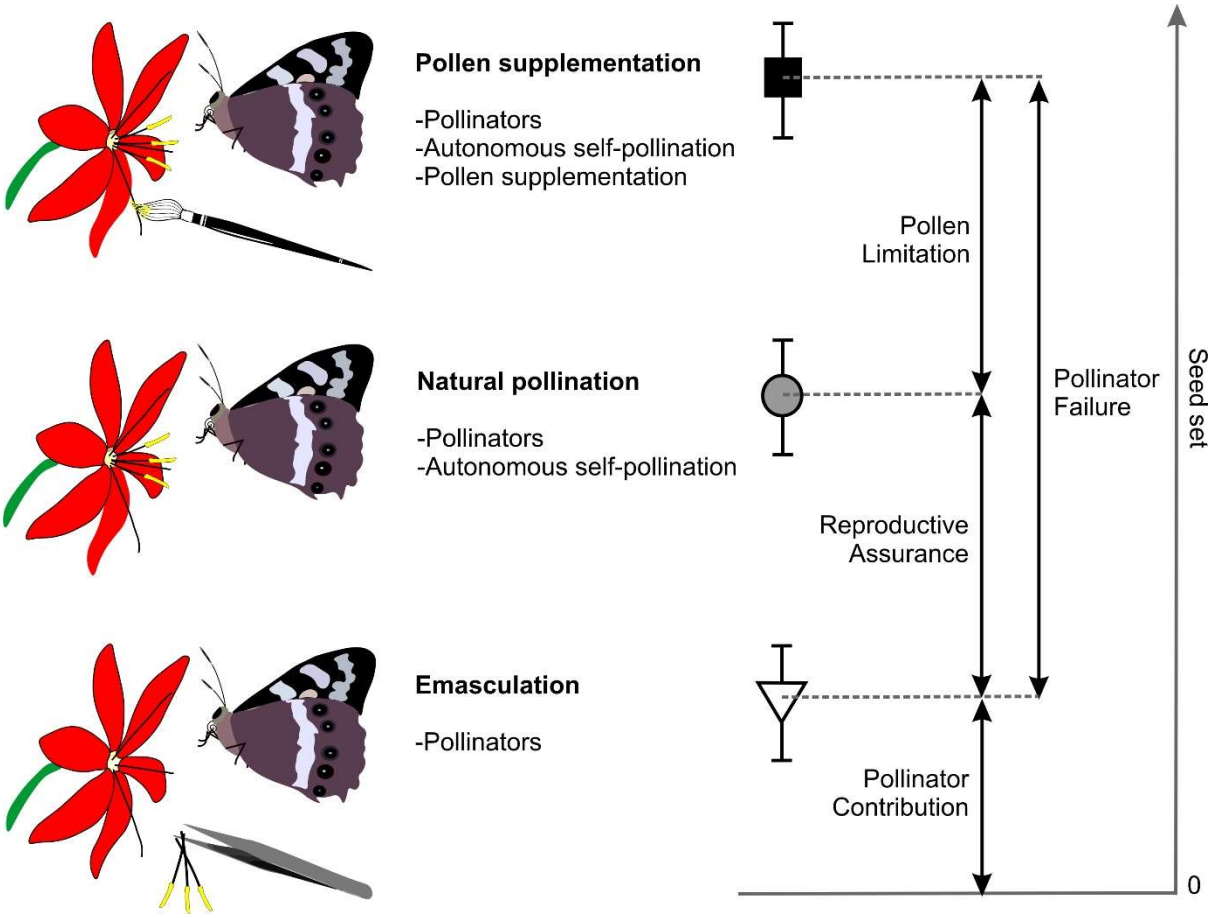

Fig. 5.2 Schematic diagram illustrating experimental design, pollen sources for each treatment and the differences between treatments used to quantify pollinator failure, reproductive assurance and pollen limitation of fecundity.

Figure 5.3

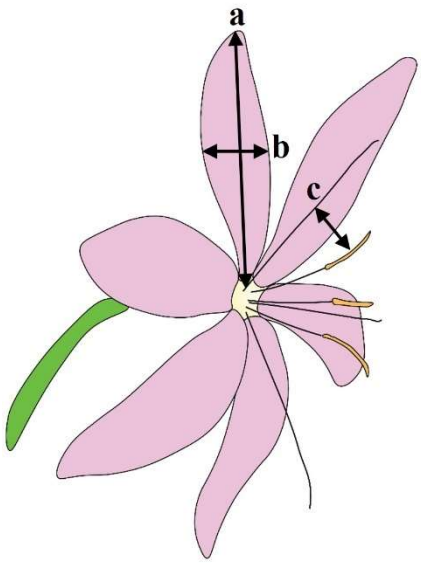

Fig. 5.3 Diagram of a *H. coccinea* flower showing floral measurements to quantify floral size (tepal length, a; tepal width, b) and herkogamy, c.

Figure 5.4

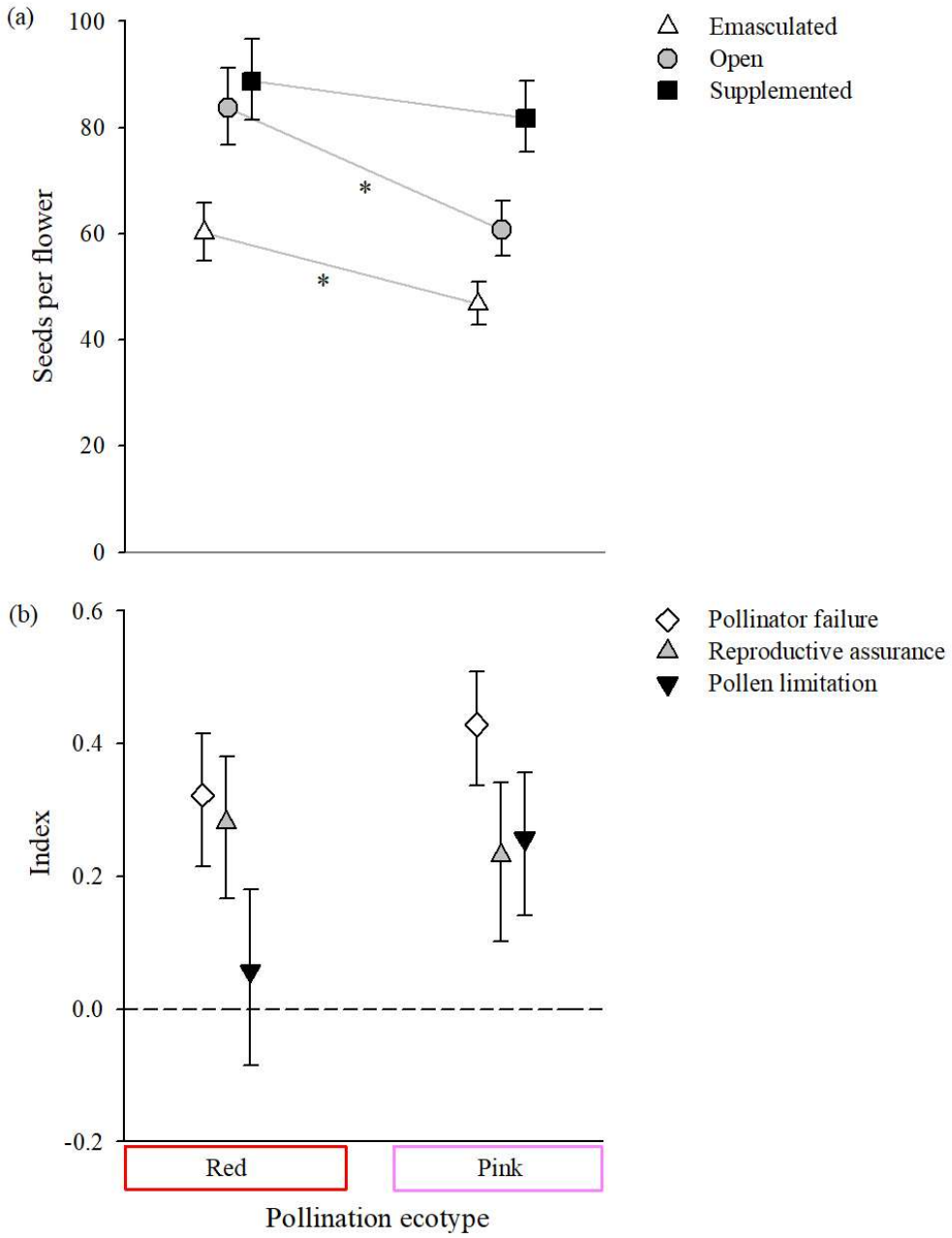

Fig. 5.4 Pollinator failure, reproductive assurance benefit of autonomous self-fertilisation and pollen limitation in two pollination ecotypes of *H. coccinea*. (a) Reproductive success of naturally-pollinated emasculated, intact and pollen-supplemented flowers and (b) the corresponding indices of pollinator failure, reproductive assurance and pollen limitation. Symbols show mean ± SE (a) or 95% confidence limits (b). Index values for which the 95% confidence limits do not overlap zero are considered significant. Results of associated statistical tests are shown in Table 5.1.

Figure 5.5

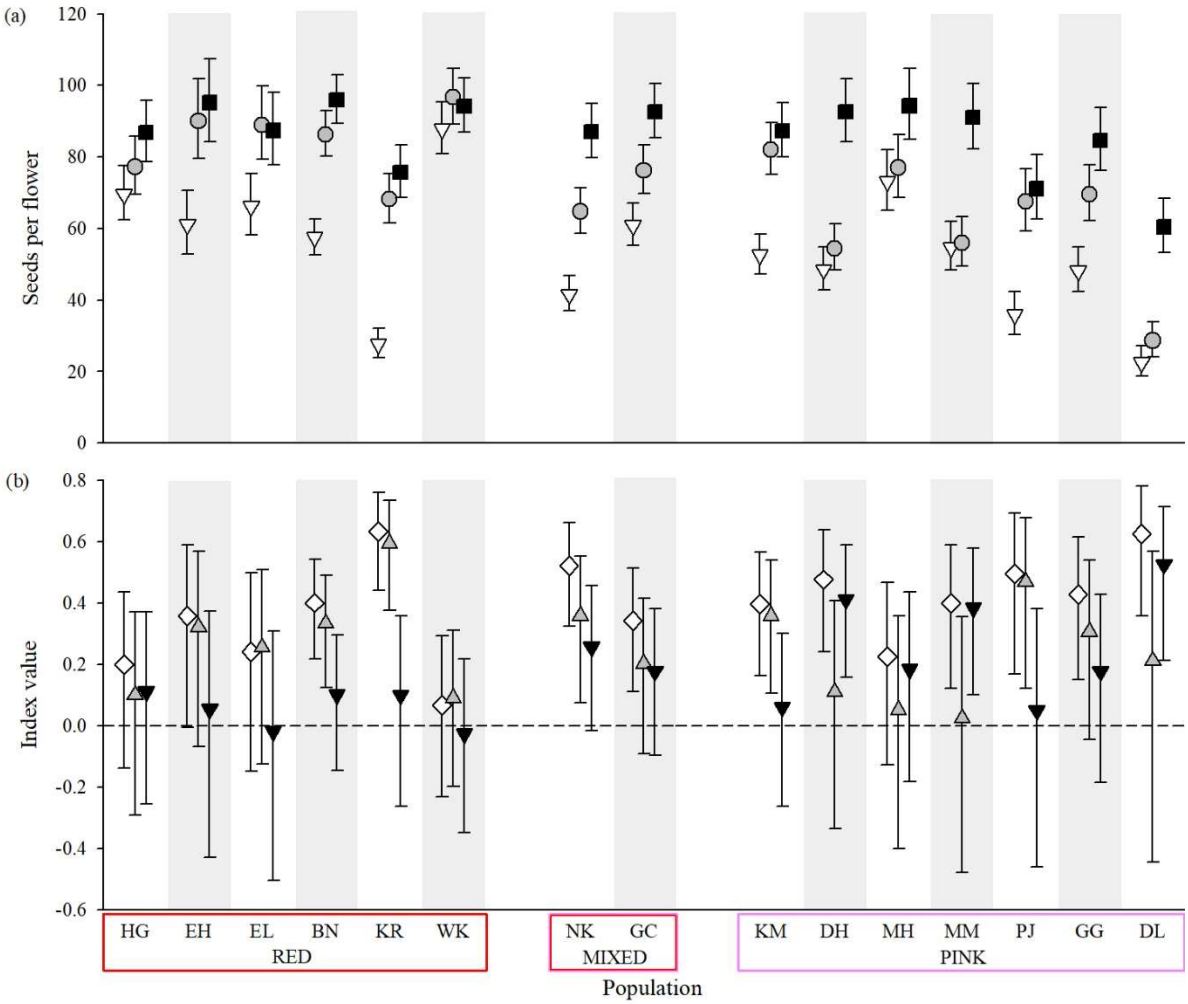

Fig. 5.5 Variation among 15 *H. coccinea* populations during 2012 in (a) mean ± SE seed production by naturally-pollinated emasculated (white upward triangles), intact (grey circles) and pollen-supplemented (black squares) flowers, and (b) corresponding mean ± 95% CI indices of pollinator failure (white diamonds), reproductive assurance (grey upward triangles) and pollen limitation (black downward triangles). Indices for which the 95% confidence intervals exclude zero are considered significantly different from zero. Results of associated statistical tests are shown in Table 5.1.

Figure 5.6

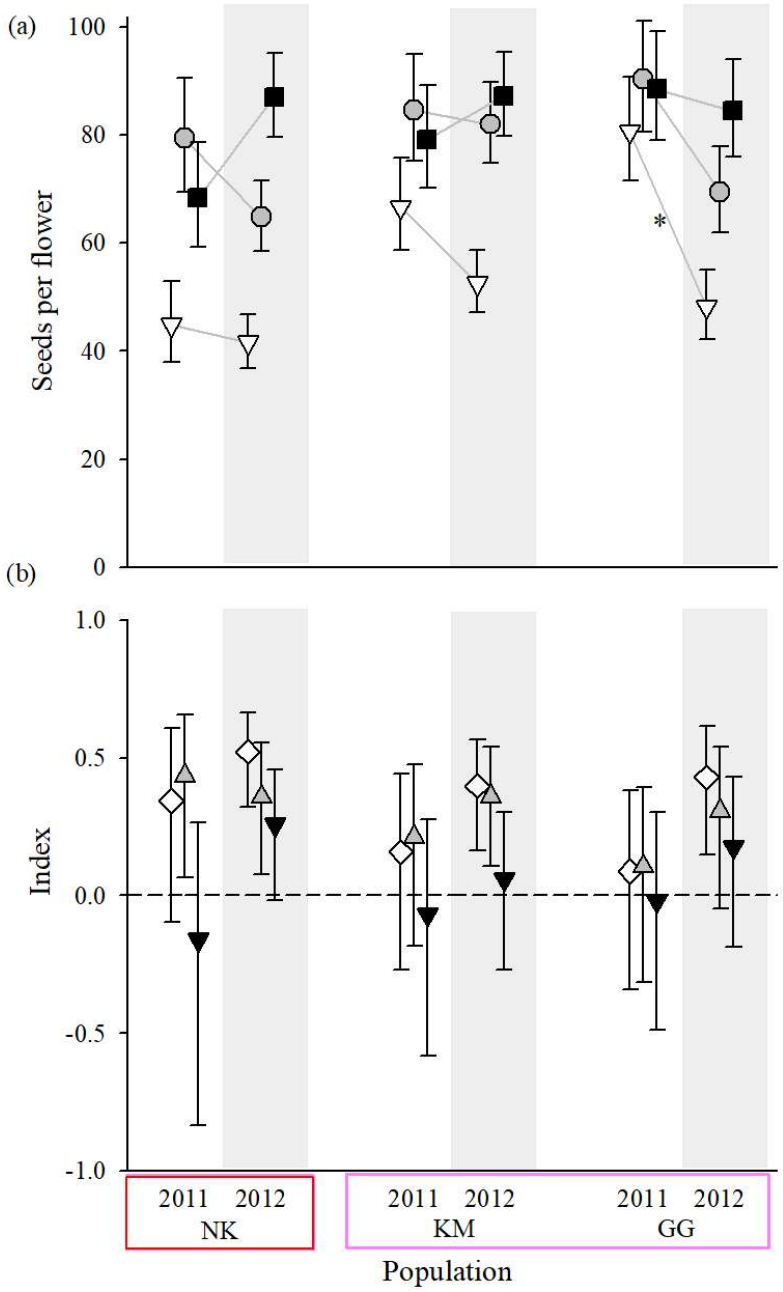

Fig. 5.6 Variation among three *H. coccinea* populations during 2011 and 2012 in (a) mean ± SE seed production per flower by emasculated flowers (downward triangles) and intact flowers (circles), and (b) the mean ± 95% CI indices of pollinator failure (white diamonds), reproductive assurance (grey upward triangles) and pollen limitation (black downward triangles). Indices for which the 95% confidence intervals exclude zero are considered significantly different from zero. Results of associated statistical tests are shown in Table 5.1.

Figure 5.7

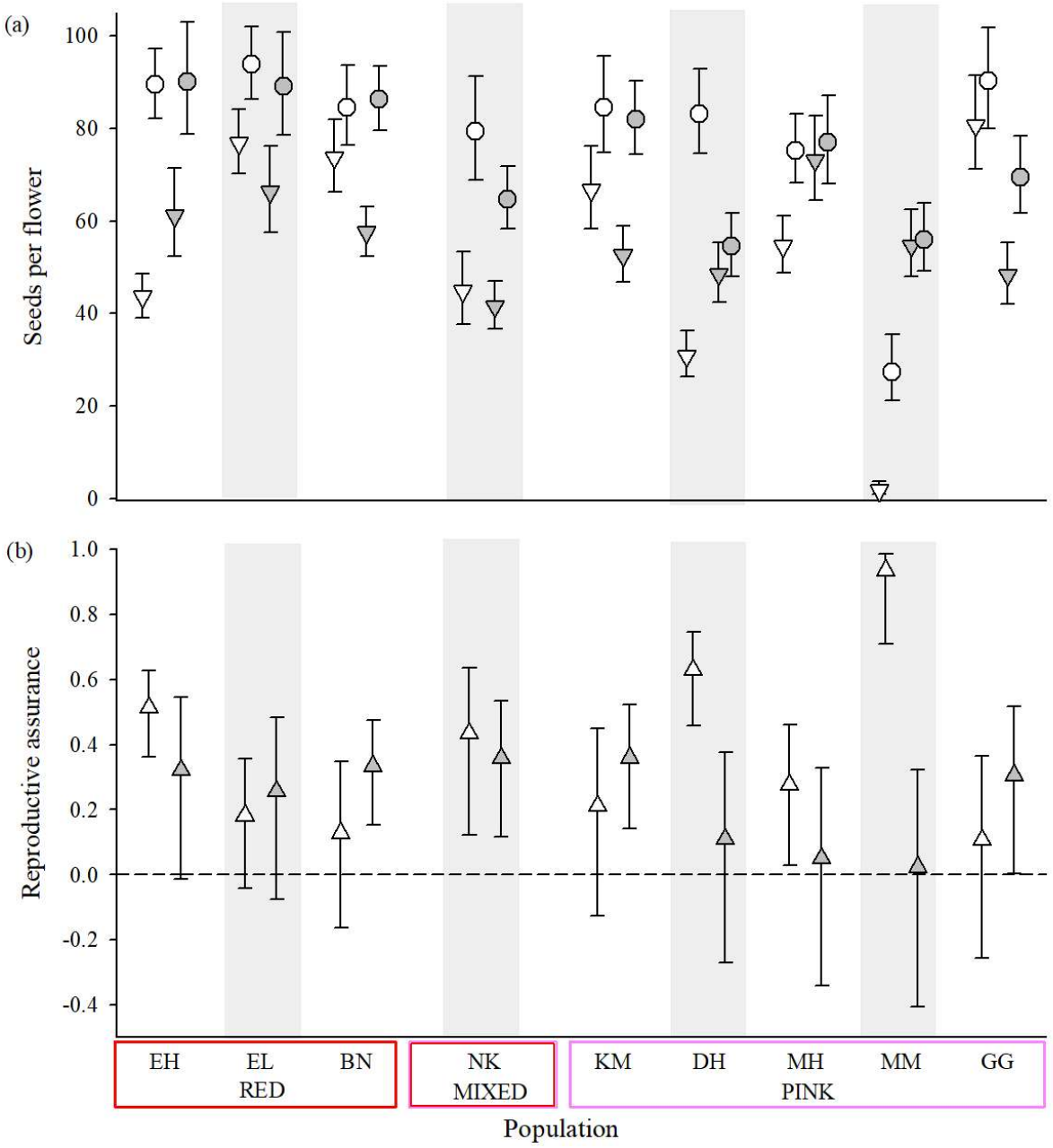

Fig. 5.7 Variation among nine *H. coccinea* populations during 2011 (open symbols) and 2012 (filled symbols) in (a) mean ± SE seed production per flower by emasculated flowers (downward triangles) and intact flowers (circles), and (b) mean ± 95% CI indices of reproductive assurance. Reproductive assurance means for which the 95% confidence interval excludes zero indicate a significant contribution of autonomous self-fertilisation to seed production. Results of statistical tests are shown in Table 5.2

Figure 5.8

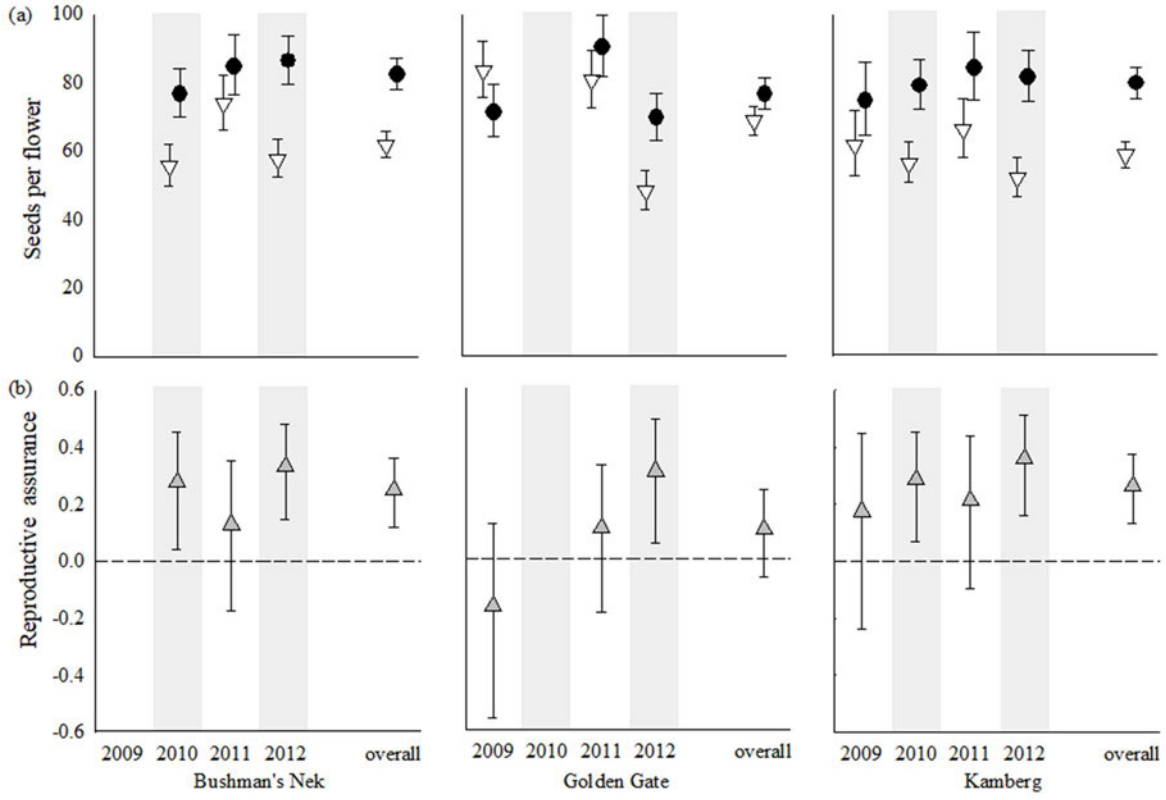

Fig. 5.8 Variation among three or four reproductive seasons for three *H. coccinea* populations in (a) mean ± SE seed production per flower for emasculated flowers (downward triangles) and intact flowers (circles), and (b) mean ± 95% CI index of reproductive assurance. Reproductive assurance means for which the 95% confidence interval excludes zero indicate a significant contribution of autonomous self-fertilisation to reproductive success. Results of statistical tests are shown in Table 5.2

Figure 5.9

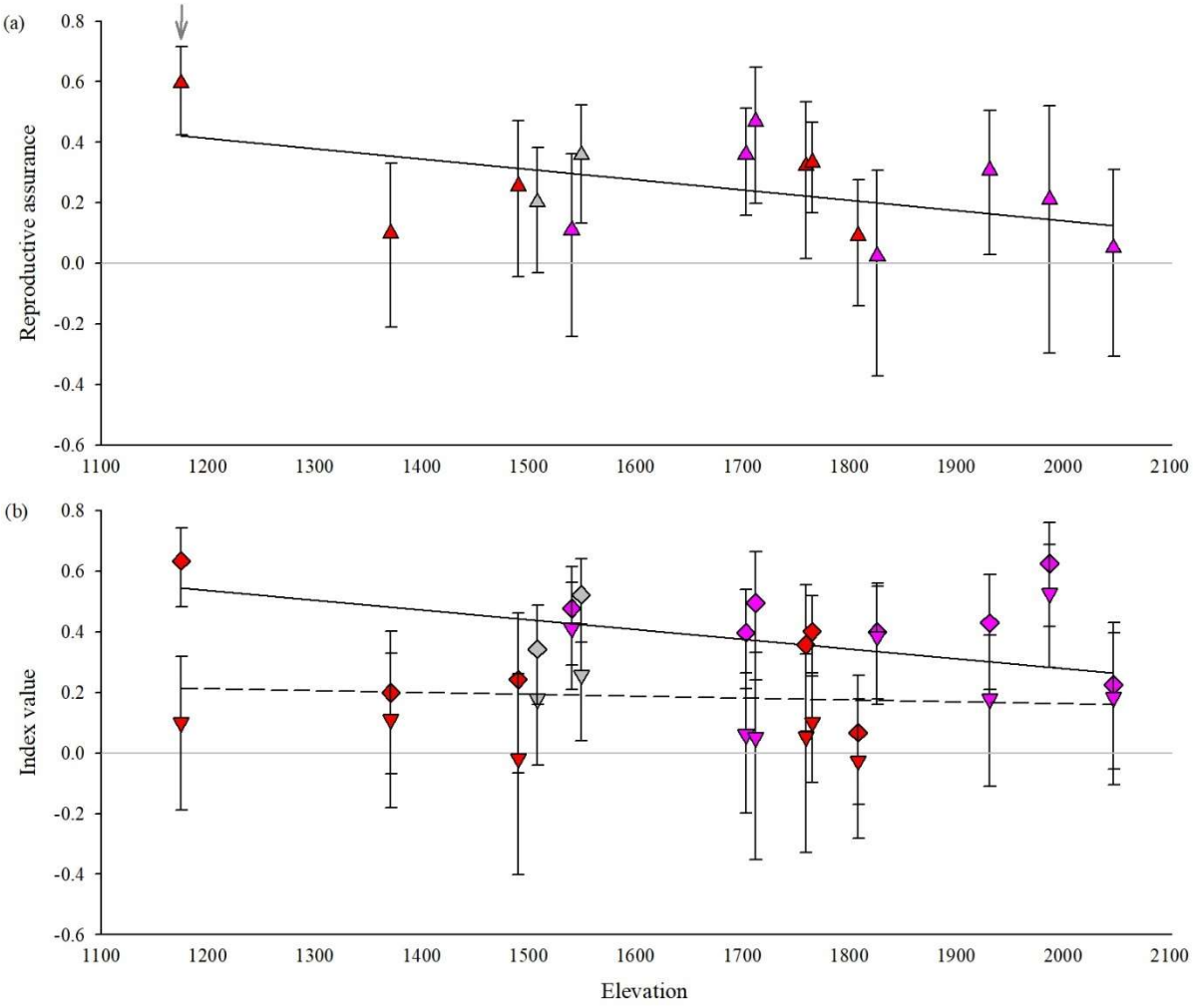

Fig. 5.9 Relations of the mean ± 95% CI indices of (a) reproductive assurance (upward triangles) and (b) pollinator failure (diamonds, solid line) and pollen limitation (downward triangles, dashed line) to population elevation. Symbol colour indicates flower colour in each population, with grey representing mixed populations. Black lines represent fitted regression relations. Results of associated statistical tests are shown in Table 5.3 and in the text. The grey arrow indicates the population that determines the statistical significance of the relation of RA to elevation.

Tables

Table 5.1. Results of GLMMs assessing fixed effects on per-flower seed production for *Hesperantha coccinea*. All analyses compared the effects of pollination treatment (natural, emasculated, supplemented), but considered different subsets of populations, as indicated. The three-population analysis also assessed differences between 2011 and 2012.

Analysis	Effect	χ^2	df	*P*
Pollination ecotypes	Treatment	85.41	2	<0.001
2012, 15 populations	Ecotype	4.33	2	0.115
Population random	Treatment x Ecotype	6.29	4	0.179
Population details	Treatment	154.95	2	<0.001
2012, 15 populations	Population	153.44	14	<0.001
Population fixed	Treatment x Population	60.71	28	<0.001
2011 and 2012	Treatment	40.04	2	<0.001
BN, GG and KM	Year	4.24	1	0.039
	population	9.32	2	<0.001
	Treatment x Year	8.00	2	0.018
	Treatment x Population	3.33	4	0.504
	Year x Population	4.18	2	0.123
	Treatment x Year x Population	1.77	4	0.778

Table 5.2. Results of GLMMs contrasting seed set for naturally pollinated and emasculated flowers during multiple flowering seasons.

Analysis	Effect	χ^2	df	*P*
Nine populations during two years	Treatment	66.95	1	<0.001
	Year	8.69	1	0.003
	Population	80.07	8	<0.001
	Treatment x Year	11.19	1	0.001
	Treatment x Population	17.42	8	0.026
	Year x Population	44.13	8	<0.001
	Treatment x Year x Population	28.93	8	<0.001
Kamberg (KM) (2009 - 2012)	Treatment	13.67	1	<0.001
	Year	1.56	3	0.668
	Treatment x Year	1.47	3	0.688
Bushman's Nek (BN) (2010, 2011, 2012)	Treatment	12.73	1	<0.001
	Year	3.47	2	0.177
	Treatment x Year	1.90	2	0.387
Golden Gate (GG) (2009, 2011, 2012)	Treatment	1.56	1	0.211
	Year	14.08	2	<0.001
	Treatment x Year	6.00	2	0.050

Appendix

Table A5.1 Geographic co-ordinates (UTM), elevation (m asl) and numbers of plants included in pollination experiments from 2009 to 2012 in 15 populations of *Hesperantha coccinea*. Bold type indicates experiments that included the pollen supplementation treatment in addition to emasculated and naturally pollinated flowers.

Flower colour	Population	Population code	S	E	Elevation	2009	2010	2011	2012
Red	Bushman's Nek	BN	-29.843	29.209	1765		29	22	**35**
Red	Elands Heights	EH	-30.818	28.207	1759			31	**12**
Red	Elliot	EL	-31.313	27.867	1490			30	**14**
Red	Hogsback	HG	-32.485	26.950	1371				**19**
Red	Karkloof	KR	-29.318	30.171	1175				**22**
Red	Wakkerstroom	WK	-27.309	30.231	1808				**27**
Mixed	Giants Castle	GC	-29.223	29.548	1508				**27**
Mixed	Nkolweni	NK	-29.507	29.717	1549			**12**	**25**
Pink	Devil's Hoek	DH	-28.714	28.934	1540			19	**20**
Pink	Dullstroom	DL	-25.414	30.112	2013				**16**
Pink	Golden Gate	GG	-28.452	28.759	1713	16		**15**	**18**
Pink	Kamberg	KM	-29.38	29.659	1703	11	26	**15**	**25**
Pink	Mahai	MH	-28.696	28.906	2047			25	**16**
Pink	Metsimatso	MM	-28.592	28.919	1826			8	**18**
Pink	Puthadjitjaba	PJ	-28.528	28.672	1771				**14**

Table A5.2 Trait means, sampling, and results of ANOVAs for number of flowers per inflorescence, flower size and herkogamy for 15 study populations. Numbers in parentheses indicate the number of plants (Inflorescence size) or flowers (Flower size and herkogamy) sampled in each population.

Trait	Display	Flower size (mm)	Herkogamy (mm)
Population		Mean ± SE (n)	
BN	7.6 ± 0.2 (67)	447.0 ± 8.6 (41)	12 ± 1.0 (27)
DH	12.9 ± 0.9 (20)	191.9 ± 7.9 (20)	3.3 ± 0.6 (6)
DL	9.8 ± 0.4 (33)	468.2 ± 17.5 (17)	9.6 ± 1.2 (18)
EH	8.6 ± 0.6 (12)	359.8 ± 16.3 (15)	6.2 ± 1.9 (8)
EL	10.9 ± 0.4 (40)	334.9 ± 12.2 (35)	7.8 ± 1.2 (20)
GC	10.6 ± 0.5 (51)	168.0 ± 8.3 (27)	5.7 ± 1.0 (24)
GG	10.9 ± 0.5 (54)	243.1 ± 8.5 (29)	8 ± 1.1 (19)
PJ	12.6 ± 0.6 (32)	240.6 ± 7.5 (20)	13.0 ± 1.4 (11)
HG	8.7 ± 0.4 (40)	325.5 ± 12.4 (22)	14.0 ± 1.1 (23)
KM	10.9 ± 0.4 (54)	205.0 ± 7.1 (54)	4.7 ± 0.7 (43)
KR	10.9 ± 0.7 (20)	280.5 ± 9.3 (31)	5.9 ± 0.4 (31)
MH	11.8 ± 0.6 (21)	245.3 ± 7.2 (46)	8.1 ± 1.6 (26)
MM	10 ± 0.5 (30)	240.8 ± 8.0 (30)	11.9 ± 1.3 (30)
NK	12.1 ± 0.7 (25)	251.1 ± 13.2 (25)	6.1 ± 0.6 (25)
WK	10.4 ± 0.4 (30)	437.0 ± 10.3 (30)	5.9 ± 0.7 (30)
Total n	529	442	341
		ANOVA results	
df	14, 343	14, 441	14, 528
F	8.8	88.9	8.4
Significance	***	***	***

Table A5.3 Results of generalised linear models assessing the effect of floral emasculation on floral fecundity, longevity and pollinator attraction.

Experiment	Effect	χ^2	d.f.	*P*
Longevity	Treatment	0.685	1	0.408
Fecundity	Treatment	0.050	1	0.822
	Population	3.989	3	0.263
	Treatment x population	4.342	3	0.227
Pollinator attraction: 1st choices	Pollinator species	2.026	2	0.363
Pollinator attraction: all visits	Pollinator species	1.116	2	0.572

Table A5.4 Results of contrasts of seed set between years for emasculated and for naturally pollinated intact flowers in nine populations.

Flower colour	Population	Emasculated		Intact	
		t	*P*	*t*	*P*
Red	EH	-1.78	0.076	-0.05	0.963
Red	EL	0.89	0.372	0.35	0.725
Red	BN	1.76	0.079	-0.15	0.880
Mixed	NK	-4.65	<.0001	1.16	0.247
Pink	KM	1.35	0.178	0.20	0.842
Pink	DH	-2.18	0.029	2.54	0.011
Pink	MH	-1.74	0.083	-0.14	0.887
Pink	MM	-4.65	>0.001	-2.48	0.014
Pink	GG	2.76	0.006	1.54	0.125

Table A5.5 Results of GLMMs to assess associations of pollinator failure, reproductive assurance and pollen limitation with geography (population elevation and latitude).

Analysis	Effect	χ^2	df	*P*
Elevation	Treatment	8.03	2	0.018
(15 populations)	Colour	7.19	2	0.027
	Elevation	2.21	1	0.137
	Treatment x Colour	11.52	4	0.021
	Treatment x Elevation	7.00	2	0.030
Elevation	Treatment	0.15	2	0.929
(14 populations, KR excluded)	Colour	1.16	2	0.560
	Elevation	0.67	1	0.415
	Treatment x Colour	12.83	4	0.012
	Treatment x Elevation	0.07	2	0.967
	Colour x Elevation	1.22	2	0.544
Latitude	Proximity	0.39	1	0.532
(proximity to range edge)	Colour	15.23	2	<0.001
	Treatment	14.90	2	0.001
	Proximity x Colour	20.05	2	<0.001
	Proximity x Treatment	2.53	2	0.283

Table A5.6 Results of GLMMs to assess population associations of pollinator failure, reproductive assurance and pollen limitation with mean floral traits (flower size, display, and herkogamy).

Analysis	Effect	χ^2	df	*P*
Number of flowers	Treatment	0.50	2	0.779
	Colour	16.75	2	0.000
	Display	2.05	1	0.152
	Treatment Colour	6.43	4	0.169
	Treatment x Display	0.62	2	0.732
	Colour x Display	17.54	2	<0.001
Flower size	Size	0.94	1	0.333
	Colour	21.11	2	<0.001
	Treatment	2.03	2	0.363
	Size x Colour	22.88	2	<0.001
	Size x Treatment	0.89	2	0.640
Herkogamy	Treatment	4.28	2	0.118
	Colour	0.67	2	0.716
	Herkogamy	0.61	1	0.434
	Treatment x Colour	6.18	4	0.186
	Treatment x Herkogamy	0.12	2	0.941
	Colour x Herkogamy	1.17	2	0.558

Figure A5.1

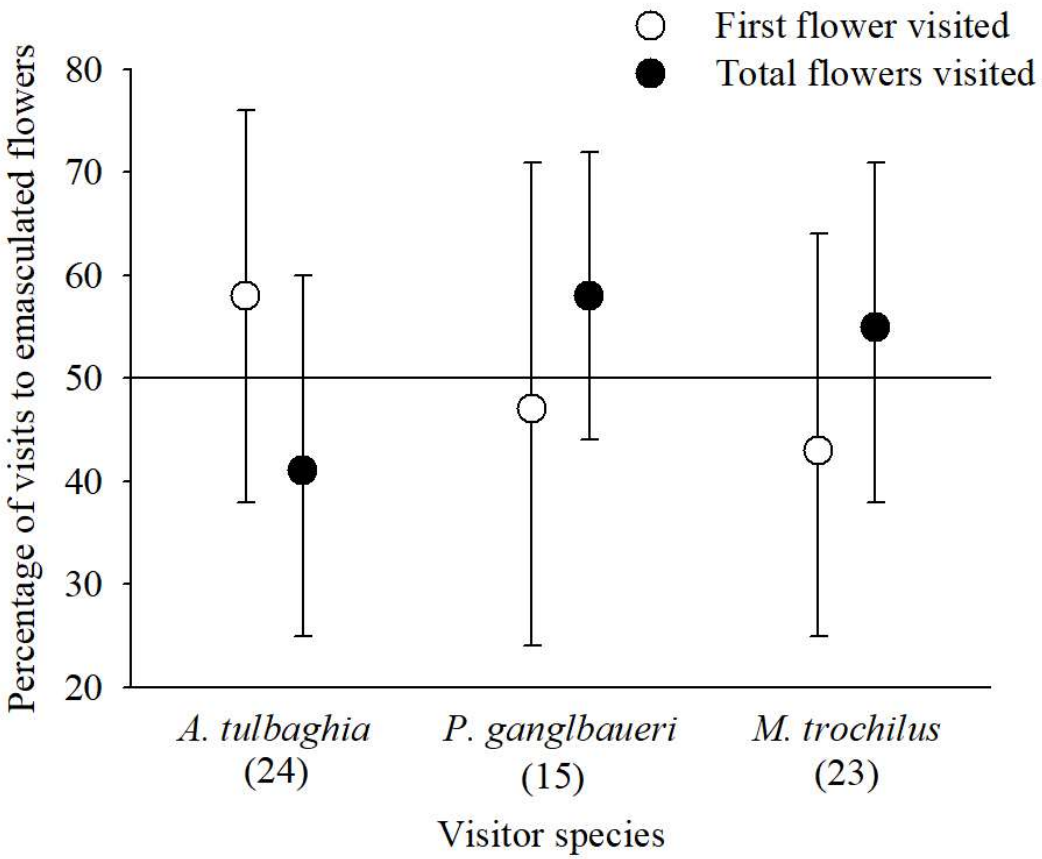

Fig. A5.1 Effect of emasculation on pollinator attraction. Symbols show mean ± 95% confidence limits for first choice preference in favour of emasculated flowers (open symbols) and total number of visits to emasculated flowers as a percentage of total visits to flowers on experimental inflorescences (filled symbols). Values for which the 95% confidence limits overlap 50% indicate no preference. Numbers in parentheses indicate the number of insect individuals of each species involved in trials. Results of associated statistical tests are shown in Table A5.1.

CHAPTER 6: CONCLUDING DISCUSSION

In this study, I have established that floral divergence among populations of *Hesperantha coccinea* reflects adaptation to different pollinators in a Best-of-Both-Worlds reproductive system. In this concluding chapter, I summarize the results of this study and discuss them first in the context of current support for the role of pollinators in floral diversification and in relation to our understanding of Best-of-Both-Worlds reproduction. The discussion on pollinator-driven divergence focusses on the underlying mechanisms and targets of selection. The discussion of Best-of-Both-Worlds reproduction considers the significance of weak inbreeding depression for maintenance of pollinator specialisation in showy selfers, and of the absence of geographic or floral traits associations with pollinator importance for stability of Best-of-Both-Worlds reproduction. Finally, I discuss implications of the evidence revealed by this study for a pollinator shift in a showy selfer species and suggest future research in the context of this novel finding.

Summary of results

The aims of this study were twofold: to test the role of pollinators in driving adaptive divergence among pollination ecotypes of *H. coccinea*; and to investigate whether inter-population variation in reproduction by *H. coccinea* is consistent with the Best-of-Both-Worlds hypothesis. In Chapter 2, I identified that divergence in floral colour, morphology, orientation and nectar sucrose content among populations correlates with pollinator differences. In particular, high-elevation, pink-flowered populations in the northern Drakensberg and Mpumalanga are visited mostly by long-proboscid flies, whereas red-flowered populations typically at lower elevation in the southern Drakensberg and Natal Midlands are visited exclusively by butterflies. Comparison of floral traits between plants *in situ* and in a common garden confirmed that trait divergence is genetically based and thus that morph differences largely reflect responses to selection, rather than plastic responses to environmental differences. Quantification of per-visit pollen deposition confirmed that flies and butterflies are effective pollinators and therefore potential agents of selection, whereas a day-flying hawk moth species is a relatively ineffective pollinator, despite visiting *H. coccinea* frequently in several populations. Colour choice tests with model flowers revealed strong preferences of flies for pink and of butterflies for red. These strongly contrasting colour preferences and the convergence of flower colour of the two morphs of *H.*

coccinea with that of other plant species that share the same fly or butterfly pollinators suggest that colour is a key trait mediating the pollinator differences between the morphs.

These results motivated the reciprocal translocation experiments reported in Chapter 3, which confirmed that floral divergence reflects adaptation to different pollination environments across the species' range. In populations of the pink-flowered morph in the northern Drakensberg and of the red-flowered morph in the southern Drakensberg, local morphs produced more seeds than foreign morphs. Thus, differences in pollination environment among populations likely underlie local adaptation. Choice tests and quantification of per-visit pollen deposition revealed that both pollinator signal preference and mechanical fit contribute to differences in pollination success and mediate the advantage of local morphs. Pollinator preferences were not affected by the local flower colour of *H. coccinea*, suggesting that colour preferences are innately determined and not a result of conditioning on local plants. Visitation in mixed arrays further demonstrated that differences in the composition of local pollinator communities, rather than varying preferences by specific pollinator species, underlie the divergence in flower colour. In addition to documenting population associations between traits and pollinators, this study verified that floral variation is locally adaptive and identified the mechanisms (signal preference and fit) and geographic basis of adaptive pollinator-mediated divergence. Together, the results of Chapters 2 and 3 supported the hypothesis that floral variation among *H. coccinea* populations reflects adaptation to functionally distinct pollinators.

In Chapters 4 and 5 the focus shifted to investigation of breeding and mating system parameters and the roles of pollinators and autonomous self-fertilisation for reproduction in *H. coccinea*. The hand-pollination experiments described in Chapter 4 demonstrated that in the absence of pollinators autonomous self-fertilisation increased fecundity in populations of both ecotypes of *H. coccinea*. Comparisons of progeny performance for plants grown from seed in a greenhouse revealed no difference in performance during early life stages and weak cumulative inbreeding depression. In contrast, in two of the three studied populations, outcrossed progeny produced more flowers than selfed progeny, suggesting that reproduction via pollinator-mediated outcrossing confers some reproductive benefit over self-fertilisation. Results of the greenhouse experiments in Chapter 4 thus established the capacity for Best-of-Both-Worlds reproduction in populations of both ecotypes, and analysis of SSR's established that populations of both ecotypes engage in mixed mating.

Chapter 5 assessed whether the capacity for Best-of-Both-Worlds reproduction was realised under natural pollination conditions in populations across the species' range. Consistent with Best-of-Both-Worlds reproduction, seed set of emasculated flowers indicated variation in pollinator service among populations and seasons, which was partially ameliorated in intact flowers by autonomous self-fertilisation. Populations of the pink-flowered ecotypes experienced lower pollinator-mediated seed set than those of the red-flowered ecotype. In addition, even though autonomous self-fertilisation increased fecundity in both ecotypes, reproduction by the pink ecotype, but not the red ecotype, was limited by pollen receipt. Finally, the absence of geographic structuring of pollinator failure and high pollinator service, even in peripheral populations, argue against evolution of selfing races at range edges or in regions characterised by pollinator failure. Instead, these results suggest that selfing is part of a stable Best-of-Both-Worlds strategy in *H. coccinea*. Absence of associations between reliance on selfing with either herkogamy, a trait which typically evolves quickly in response to selection for increased reliance on selfing, or flower size also suggest that *H. coccinea* is a showy selfer, with a combination of traits that facilitate stable Best-of-Both-Worlds reproduction.

Together, the evidence from Chapters 2 and 3 demonstrating local adaptation to contrasting pollinators and the evidence for autofertility and Best-of-Both-Worlds reproduction presented in Chapters 4 and 5, strongly identify that in *H. coccinea*, a pollinator shift occurred in spite of lack of dependence on pollinators. Evidence of weak inbreeding depression and some pollen limitation suggest that selection through both male and female components of reproductive success likely underlie maintenance of floral adaptations for pollinator specialisation in Best-of-Both-Worlds systems, in general, and may specifically have facilitated the pollinator shift in *H. coccinea*.

Mechanisms and targets of pollinator-mediated selection

Evidence from this study is important in the light of limited evidence definitively supporting the role of pollinators as drivers of flower colour variation (Rausher 2008). Many pollinator species discriminate among plant species based on flower colour (Campbell et al. 2010, Jersakova et al. 2012, Newman et al. 2012, Streinzer et al. 2019), but this behaviour must affect reproductive success to influence selection on colour, and this is seldom tested (Rausher 2008). Indeed, flower colour variation in some species has instead been attributed to

non-pollinator causes, including biotic (herbivores, parasites) and abiotic (moisture, UV) agents of selection (Strauss and Whittall 2006). Differences in flower colour have also been associated with differences in competitive ability (Warren and Mackenzie 2001) and adaptation to abiotic aspects of environments (Schemske and Bierzychudek 2007, Koski and Galloway 2020). In contrast, in Chapter 3 I demonstrated that pollinator preferences determine pollinator visitation (Fig. 3.2a), pollination success (Fig. 3.2b) and differences in fertility (Fig. 3.3). Thus, this study demonstrates that flower colour in *H. coccinea* is attributable to pollinator adaptation. Further evidence for a role of pollinator colour preferences in driving colour divergence is provided by the striking convergence in flower colour among species that share pollinators in both fly and butterfly pollination guilds (Fig. 2.7).

Pollination syndromes, similar traits in often distantly related species that share a pollinator, suggest that adaptation to different pollinators involves suites of traits (Faegri and van der Pijl 1979, Rosas-Guerrero et al. 2014). In *H. coccinea*, variation in suites of traits was observed among populations in association with pollination by different insects. In addition to flower colour, this study identified several morphological traits that differ between pollination ecotypes of *H. coccinea*, including floral tube, stamen and style lengths, petal width, floral orientation and nectar sucrose (Table 2.1). Flower colour was the only trait that was tested explicitly for its functional significance. This study demonstrated that overall morphological differences between flower morphs are important for pollen-transfer effectiveness of respective pollinators (Fig. 3.1b), but the functional significance of particular morphological traits that also differ between morphs merits further study. In particular, the roles of floral-tube length, flower orientation and the dissection of flower outline (a reflection of tepal width) should be investigated, as they differ strikingly between fly- and butterfly-pollinated morphs (Table 2.1).

Functional floral depth is expected to be particularly important for pollinator effectiveness, as it influences the alignment and contact of pollinators with floral sexual organs, promoting pollen removal and deposition (Nilsson 1988, Newman et al. 2015). Indeed, flower depth affects pollination success in other species that, like *H. coccinea*, are pollinated by *P. ganglbaueri* (Anderson and Johnson 2008, Pauw et al. 2009). As speculated in Chapter 2, the narrower petals of pink flowers may reflect benefits of dissected outlines for fly attraction (Faegri and van der Pijl 1979, Jersakova et al. 2012), whereas wider petals may improve per visit effectiveness of butterflies by reducing the frequency of visits that do not

involve anther and/or stigma contact. Flower orientation affects how pollinators approach flowers and the precision of their contact with reproductive parts (Fenster et al. 2009). Variation in pollinator positioning is more limited by vertical orientation than horizontal orientation, resulting in greater pollination accuracy for vertically oriented flowers (Ushimaru and Hyodo 2005). In *H. coccinea*, differences in floral orientation may therefore affect pollination-effectiveness of hovering flies (Muchhala 2007, Campbell et al. 2016) and/or of settling butterflies (Faegri and van der Pijl 1979, Fenster et al. 2009). A combination of methods, including trait manipulations (Campbell 2009), breeding of near-isogenic lines with contrasting traits (Bradshaw and Schemske 2003) and quantification of selection gradients (e.g. Kulbaba and Worley 2012, 2013) could be used to establish whether these traits are under selection and whether pollinators are the agents of selection. If so, further studies could evaluate evidence for these hypotheses regarding the functional significance of traits.

To implicate pollinators as selective agents, fitness gradients for traits that show a signature of selection could be compared between pollen-supplemented and open-pollinated plants to quantify the strength and nature of pollinator-mediated selection within populations (Lande and Arnold 1983, Galen 1989, Sletvold et al. 2010). Although pollen-supplementation with cross pollen can involve an unusual proportion of high-quality pollen (Aizen and Harder 2007), this is probably not problematic for *H. coccinea*, as self- and cross-pollination result in similar seed-production (Chapter 4). To establish the functional significance and mechanisms of pollinator-mediated selection on traits, experimental manipulations (Johnson and Steiner 1997, Campbell 2009) or model flowers (Chapter 2, Jersakova et al. 2012) can be used. For example, the effect of shortening the tube of *H. coccinea* flowers on fruit set differed between a fly- and a butterfly-pollinated population (Cozien unpublished). To test whether differences in petal width and flower orientation between colour morphs affect pollinator effectiveness, manipulations of these traits could be combined with comparisons of per-visit pollen deposition (Fulton and Hodges 1999, Ashman et al. 2000, Wang et al. 2014). Similarly, the significance of differences in nectar sucrose between colour forms of *H. coccinea* (Table 2.1) could be investigated by assessing the effects of replacing nectar with sugar solutions standardised to reflect average sucrose:hexose ratios of red and pink flowers, respectively, on visit duration, rates of nectar uptake and per-visit effectiveness of each pollinator. Experiments with nectar sucrose would be particularly interesting in the light of widespread associations between pollination system and differences in nectar composition (Baker and

Baker 1983) and recent evidence for pollinator-mediated selection on nectar sugar composition (Gijbels et al. 2014).

Finally, anecdotal evidence for convergence of floral traits, such as floral orientation (Johnson and Bond 1994) and nectar sucrose (Goldblatt et al. 2004, Goldblatt and Manning 2006), among species that share the same pollinator could be extended. Specifically, statistical comparison of traits within guilds to those of non-guild sister taxa or congeners could provide a macroevolutionary test of the hypothesis that these traits reflect adaptation to different pollinators (Shuttleworth and Johnson 2012, Jürgens et al. 2013).

Implications of weak inbreeding depression for BOBW reproduction and showy selfer flowers

Inbreeding depression can play a critical role in maintaining outcrossing in mixed mating systems (e.g. Eckert et al. 2006), and may specifically facilitate selection for outcrossing traits in Best-of-Both-Worlds systems (Goodwillie et al. 2005, Goodwillie and Weber 2018, also see Morgan and Wilson 2005). Strong inbreeding depression erodes the reproductive assurance benefit of selfing and provides a clear explanation for maintenance of outcrossing and adaptations for pollinator-mediated reproduction in some systems (e.g. Herlihy and Eckert 2002, Dart and Eckert 2013a). In contrast to this expectation, weak inbreeding depression, as found for *H. coccinea* (Chapter 4), is not unusual in species in which reproduction is otherwise largely consistent with Best-of-Both-Worlds condition (e.g. Carrio et al. 2008, reviewed in Goodwillie and Weber 2018). As discussed in Chapter 4, this association contradicts the dominant conception that Best-of-Both-World reproduction requires strong inbreeding depression (e.g. Goodwillie and Weber 2018). This incongruity emphasises that additional factors, such as the siring advantages of outcrossing, likely also influence the evolution of Best-of-Both-Worlds reproduction and associated trait combinations.

Does selection through siring success underlie the showiness of showy selfers?

As outcrossing depends on successful pollen dispersal, the effects of floral traits on male function should strongly influence floral evolution (Bell 1985, Burd and Callahan 2000). The

relevance of male function to selection has long been recognised (Stanton et al. 1992, Snow and Lewis 1993), but most studies have quantified female components of plant fitness, in part because it is easier to measure, as was the case in this study of *H. coccinea*. The development of molecular markers has allowed more accurate measurement of selection through male function (e.g. Morgan and Conner 2001, Hodgins and Barrett 2008). The SSR markers developed for *H. coccinea* (Wolff et al., 2009) and used to quantify outcrossing rates in this study (Chapter 4) are ideal for paternity analysis and would enable quantification of selection through male function (Kulbaba and Worley 2012, Gleiser et al. 2014). SSR's could also be used to explore whether higher paternal diversity in pink-flowered populations than in red-flowered populations of *H. coccinea* (Chapter 4) reflects effects pollinator identity (fly versus butterfly) on variation in siring success (cf. Rhodes et al. 2017). Recent developments that allow pollen tracking could also be applied to compare pollen dispersal between morphs of *H. coccinea* (Minnaar and Anderson 2019). Either of these methods could be used to quantify phenotypic selection through male mating success in reciprocal translocation experiments, as implemented in Chapter 3 for female success, but with non-emasculated flowers (Kulbaba and Worley 2013). Such experiments would also provide useful insight into the relative strength of selection through male and female components of plant fitness in a Best-of-Both-Worlds reproductive system.

Pollinator shifts as drivers of floral diversity

Local adaptation to different pollinators, as demonstrated in this study for *H. coccinea*, is thought to underlie much of angiosperm floral diversity (Grant and Grant 1965, Stebbins 1970). Several interspecific patterns indicate a role for adaptation to different pollinators in floral diversification, including coincidence of pollinator shifts with changes in floral traits and speciation (Whittall and Hodges 2007, Valente et al. 2012, van der Niet and Johnson 2012) and trait similarities among species in difference clades with similar pollinators (Vogel 1954, Faegri and van der Pijl 1979, Fenster et al. 2004, Johnson 2010, Rosas-Guerrero et al. 2014). These patterns are interpreted as the products of divergent evolution within lineages and convergent evolution among lineages, respectively (Vogel 1954, Faegri and van der Pijl 1979, van der Niet and Johnson 2012).

Geographic evidence is required to demonstrate divergent selection or local adaptation to contrasting pollinator environments (reviewed in Herrera, Castellanos & Medrano, 2006, but see Totland 2001). Co-variation of floral traits and pollination among populations, as shown in Chapter 2, is consistent with local adaptation to different pollination environments (reviewed in van der Niet et al. 2014a). However, floral traits are typically under selection from diverse agents, including herbivores and abiotic factors (Strauss and Whittall 2006, Ramos and Schiestl 2019). Indeed, divergence of floral traits in association with differences in pollination can arise from adaptation to local abiotic conditions (Streisfeld and Kohn 2007). Tests of adaptation to local pollinators, such as the translocation experiments presented in Chapter 3, are therefore crucial to implicate pollinators as drivers of adaptive divergence (Boberg et al. 2014, Sun et al. 2014). Contrasting adaptation among populations has been shown to reflect geographic variation in pollinator preference (Newman et al. 2012), pollinator assemblages (Gómez et al. 2009) and plant community context (Grossenbacher and Stanton 2014).

Pollination is expected to be a key driver of divergence in florally variable lineages (Stebbins 1974, Carson 1985, Johnson 1996), but studies of several such lineages in southern Africa, including in the Iridaceae, have suggested that abiotic heterogeneity, rather than pollination, has been the primary driver of diversification (Goldblatt and Manning 1996a, Goldblatt and Manning 2006, Schnitzler et al. 2011, Valente et al. 2012, but see van der Niet and Johnson 2009). The genus *Hesperantha* exemplifies this contention: its flowers vary extensively in morphology, colour, opening time and scent, which suggests that pollinators are key drivers of variation (Goldblatt et al. 2004). However, the taxonomy of this genus has been based on corm morphology, suggestive that adaptation to edaphic factors is also important (Goldblatt 2003). In this context, it is interesting that intraspecific divergence in *H. coccinea* is pollinator-driven and apparently occurred without differences in abiotic environment. This pattern contrasts with suggestions that pollinator shifts in southern African Iridaceae have reinforced prior adaptation to differences in the abiotic environment (Goldblatt and Manning 1996a, Goldblatt and Manning 2006, but see Forest et al. 2014).

Are pollination ecotypes in *H. coccinea* incipient species?

Understanding of how a particular process of divergence within species may generate macroevolutionary diversification, requires evidence such as discontinuous trait variation or

partial reproductive isolation, to show that divergent populations indeed represent forms in the process of becoming fully fledged species (Mayr 1942, Coyne and Orr 2004 , de Queiroz 2005). Although a gradient of variation is often observed in ecotypic divergence (Stebbins 1970), the occurrence of intermediate and mixed *H. coccinea* populations precludes recognition of two subspecies corresponding to the red and pink-flowered forms. These forms were originally described as separate species (Klatt 1867, Baker 1892), as they also differ in other floral traits, and were thought to have disjunct distributions in the southern and northern Drakensberg regions, respectively (Baker 1896). However, recognition that plants with flowers of both colours also occur within populations, and that populations of both pink- and red-flowered plants occur throughout the Drakensberg resulted in both species being combined into *H. coccinea* (Letty and Dyer 1962, Goldblatt 1993, Goldblatt and Manning 1996b). Furthermore, similar rates of fertilisation, and seed production following within- and between-morph crosses (Chapter 3) demonstrate the absence of postzygotic barriers between morphs of *H. coccinea*. Pollinator preferences can mediate pre-zygotic isolation (Ramsey et al. 2003a). However, in *H. coccinea*, pollinator isolation in secondary sympatry would likely be incomplete due to the propensity of butterflies to visit pink flowers occasionally (Table 2.2). Therefore, explicit assessment and comparison of the various components of reproductive isolation are still required (Ramsey et al. 2003b, Lowry et al. 2008a, Whitehead and Peakall 2014).

Specialization increases the potential of pollinators to contribute to reproductive isolation of their associated plant species, but pollinator specificity alone is unlikely to be the main basis of reproductive isolation (Waser 2001), with the possible exception of some sexually deceptive orchids (Whitehead and Peakall 2014). Isolation is more likely to evolve when differences in pollination accompanies other habitat differences (reviewed in Kay and Sargent 2009) and when multiple traits are under selection (Nosil et al. 2009). However, gene flow may be restricted due to geographic discontinuities in species distributions, resulting in effective isolation between ecotypes and enabling divergence and the eventual build-up of genetic incompatibilities (Johnson 2006). As *H. coccinea* occupies only riparian habitats, populations tend to be separated by large distances. Thus, gene flow may be reduced between populations in different river basins (e.g. Ellis et al. 2007), especially if pollen and seed flow is limited relative to distances between populations (e.g. Barrett et al. 2004, see also Johnson, 2006). Even if some gene flow does occur, this may not impede divergence (Nosil, 2008). The evidence for local adaptation in *H. coccinea* presented in Chapter 3 suggests that

selection probably overwhelms gene flow in this species. Detailed investigation of population genetics in *H. coccinea* could reveal the extent of gene flow among populations and ecotypes and the roles of topography, geographical distance and pollination in determining gene flow patterns and divergence (Lowry 2012, e.g. Cooke et al. 2014). Intriguingly, preliminary results using SSR markers (Chapter 4) suggest F_{st} differences among *H. coccinea* populations similar to those between fully differentiated species, rather than among populations within a species (Cozien et al. unpublished, Holsinger and Weir 2009). Coincidence of ecotype boundaries with genetic boundaries, would verify that red and pink ecotypes function as separate metapopulations, one criterion for incipient speciation (de Queiroz 2005, e.g. Lowry et al. 2008b).

The direction and number of pollinator shifts

As *H. coccinea* is the only *Hesperantha* species with red flowers, it seems likely that the shift to red occurred within *H. coccinea*, although the pattern of colour variation and morph distribution among populations could represent multiple origins of either or both colour forms or a reversion to pink flowers. Several *Hesperantha* species (*H. scopulosa*, *H. grandiflora*, *H. woodii*, *H. curvula, H. huttonii*), most of which co-occur in montane grassland habitats with *H. coccinea*, have flowers with similar morphology to the pink-flowered morph (narrow tepals and elongated floral tubes) and are thought to be pollinated by long proboscid flies (Goldblatt et al. 2004). Therefore, the single instance of evolution of red flowers in the genus, in association with a shift to butterfly pollination, likely represents the derived pollination system (Goldblatt and Manning 1996b, Goldblatt et al. 2004, Goldblatt and Manning 2006)).

Sequence data could be profitably applied to resolve the direction and frequency of the colour shift in *H. coccinea*. Shift frequency and directionality have been shown in intraspecific phylogenetic studies (Whittall and Hodges 2007, Valente et al. 2012). However, reticulate intraspecific trees are considered inappropriate by some to reconstruct relationships, as they indicate ongoing gene flow and persistence of ancestral polymorphisms (Smouse 1998, Posada and Crandall 2001). Within species, network-based approaches that allow multifurcate branching (Pleines et al. 2009) and combined population genetic/phylogenetic methods applicable to multiple accessions per population from the full range of a species using multiple, congruent chloroplast and nuclear markers have enabled

inferences regarding frequency and directionality of trait evolution among populations (Pettengill and Moeller 2012, Briscoe Runquist et al. 2014, van der Niet et al. 2014b).

The distribution of colour morphs across the range of *H. coccinea*, specifically with three centres of pink-flowered populations in the highest elevation regions of the species range, separated from each other by red-flowered populations (Fig. 2.1), suggests multiple colour shifts. Lower pollinator service in populations of the pink-flowered ecotype (Chapter 5) suggests that the shift to butterfly pollination involved selection to alleviate pollinator failure as pink-flowered plants colonised lower elevation habitats in which butterfly pollinators were dominant. Such directional colonisation may have been facilitated by downstream dispersal of the hydrochorous seeds of *H. coccinea* (Wagner and Goldblatt 1984, Goldblatt and Manning 1996b). Population genetic analyses could establish the direction of the shift(s) in *H. coccinea* (van der Niet et al. 2014b, Castañeda-Zárate et al. 2020) and test whether the current centres of pink and red populations represent parallel independent origins of the respective ecotypes (Soria-Carrasco et al. 2014) or inter-watershed colonization (Koski and Galloway 2020).

Pollinator shift in a showy selfer: Evaluation of potential selective mechanisms

As outlined in Chapter 1, adaptation to a novel pollinator by an autofertile species that needs pollinators to reproduce requires specific conditions. In particular, Chapters 2 and 3 document evidence for divergence in a suite of floral traits including flower colour and morphology in association with pollination by long-proboscid fly and butterfly pollinators, consistent with an adaptive pollinator shift in *H. coccinea*. In addition, Chapters 3 and 4 conclusively demonstrate that populations of both ecotypes reproduce by Best-of-Both-Worlds combinations of autonomous self-fertilisation and pollinator-mediated fecundity. Here, I evaluate which of the pathways described in the Introduction may maintain adaptations for pollinator-mediated outcrossing *H. coccinea*, and speculate on their possible contributions to the adaptive pollinator shift, based on the results described in this thesis.

Hesperantha coccinea likely experiences selection through female function, despite capacity for autonomous self-fertilisation. Equivalent seed set of self and outcross pollination in populations of both ecotypes (Table 4.2, Figure 4.3) suggests that pollen quality does not underlie any selective advantage of pollinator-mediated reproduction in either ecotype. Differences in the capacity for autonomous self-fertilisation were observed between ecotypes,

and could contribute to pollen limitation in the pink ecotype. Although red-flowered populations set similar proportions of seed via autonomous self-fertilisation and cross pollination (autofertility index = 0.89 and 0.83), populations of the pink-flowered ecotype exhibited lower capacity for autonomous self-fertilisation (0.54 and 0.72). Correspondingly, pink-flowered populations experienced significant pollen limitation. Thus, pollen limitation and selection through female function may contribute to adaptive maintenance of floral traits that facilitate pollinator interactions, especially in the pink ecotype.

Inbreeding depression estimates also suggest potential for selection through female function (Chapter 4). Despite absence of detectable inbreeding depression during earlier life stages and low cumulative inbreeding depression for populations of both colour forms, selfed progeny produced fewer flowers than outcrossed progeny. This difference suggests that pollinator-mediated outcrossing may confer a selective advantage for both ecotypes. However, this advantage may again be less important in red-flowered populations. In one of the studied red-flowered populations cumulative inbreeding depression was negligible (Table 4.1) and it was not specifically apparent for flower production (Fig. 4.3). Based on self-compatibility, capacity for autonomous self-fertilisation, pollen limitation and inbreeding depression selection through female function may contribute to selective maintenance for pollinator-mediated reproduction in both ecotypes of *H. coccinea*, despite of capacity to reproduce independently of pollinators.

Estimates of these parameters indicate that selection through female function may be stronger in populations of the pink-flowered ecotype than in those of the red-flowered ecotype. The significant contribution of pollinators to female fecundity (75% of seeds, Chapter 5) suggests ample opportunity for pollinator selection through variation in siring success among individuals. Curiously, male-mate diversity was higher in the pink-flowered population than in the red-flowered population, in contrast to the expectation of greater importance of male competition in the red-flowered populations. In red-flowered populations, male success should be emphasised due to lack of variation in female success (c.f. Bell 1985) due to higher autofertility in red-flowered populations (Chapter 4), very high pollinator activity that often eliminated pollinator limitation of reproductive success (Chapter 5) and demonstrated capacity of autofertility to eliminate pollen limitation when it does occur (Chapter 5). These results suggest that both male and female components of selection contribute to adaptations for pollinator specialisation in both ecotypes, although the absence of pollen limitation in the red-flowered ecotype underscores the need for measurement of the

role of siring success in maintaining adaptions for butterfly pollination in *H. coccinea*. If, as speculated above, red flowers are derived in *H. coccinea*, the shift may have been driven by selection to alleviate pollen limitation as pink-flowered plants as they colonised sites where butterflies were more common.

Do the conditions for pollinator shifts found in this study apply more generally?

The evolution of selfing is often associated with reduced diversification rates and often considered an evolutionary dead end (Stebbins 1957, Takebayashi and Morrell 2001, Igic and Busch 2013, Gamisch et al. 2015, Cheptou 2019), but that scenario may apply mainly to lineages in which a selfing syndrome associated with predominant autogamy has evolved (Barrett 2013). Shifts from predominant selfing to increased reliance on pollinator-mediated outcrossing are known to occur (Armbruster 1993) and high speciation rates in self-compatible lineages suggests that partial selfing can be maintained (Goldberg et al. 2010). Indeed, selfing may facilitate pollinator shifts. For example, Wessinger and Kelly (2018) showed that among pairs of bee- and bird-pollinated species in several families, derived pollination by hummingbirds occurs more frequently in self-compatible lineages. However, their study considered only 17 species pairs, and was based on evidence of self-compatibility, rather than the incidence of autonomous selfing, which specifically facilitates pollinator shifts (Wessinger and Kelly 2018). A test of the association between capacity for autonomous self-fertilisation and pollinator shifts requires a large, phylogenetically informed analysis of the co-occurrence of autofertility and pollinator transitions, including many more families or genera with known pollination and breeding systems, ideally with known timing of self-fertilisation. Global datasets of the occurrence of self-compatibility and autofertility have recently been assembled (Razanajatovo et al. 2019) and could be combined with existing data on pollination systems for well-studied families (Perez et al. 2009, van der Niet and Johnson 2012) to assess whether the mechanisms inferred to underlie the shift in *H coccinea* apply more broadly.

Species closely related to *H. coccinea* may represent cases of pollinator-driven divergence in autofertile systems. Pollinators have likely played an important role in diversification in *Hesperantha* (Goldblatt et al. 2004), yet, the self-compatibility and capacity for autonomous self-fertilisation in *H. coccinea* is not exceptional within the genus (Goldblatt 1984, Goldblatt et al. 2004). Among the dozen (of c. 80) *Hesperantha* species for which self-

compatibility has been quantified, only two species are self-incompatible and four are partially self-compatible, showing reduced fecundity following self-pollination. An additional autofertile species has a highly specialised beetle-pollination system, and four more species do not experience reduced fecundity when isolated from pollinators (Goldblatt 1984, Goldblatt et al. 2004, van Kleunen et al. 2008). Thus, despite floral adaptations in the genus reflecting shifts in pollination system (Goldblatt et al. 2004), self-compatibility and capacity for autonomous self-fertilisation are common, suggesting that Best-of-Both-Worlds reproduction, and shifts mediated by mechanisms similar to those that operate in *H. coccinea*, may be more widespread in the genus.

Conclusion

The evidence described in this study for a pollinator shift in a showy selfer species revealed two key insights relevant to current understanding of pollinator shifts and Best-of-Both-Worlds reproduction. First, although pollinator dependence probably promotes pollinator-driven divergence, it is not a necessary precondition for a pollinator shift. Second, factors other than inbreeding depression should also be considered to understand the adaptive maintenance of mixed mating and trait combinations in Best-of-Both-Worlds reproductive systems.

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